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Virus-Like Particle and Capsomere Vaccines against Rotavirus

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Abstract

Rotavirus is the major enteric pathogen that is responsible for more than 500,000 deaths of children worldwide annually. The use of live-attenuated oral rotavirus vaccines has significantly reduced child mortality rate in developed countries. However, the situation still remains unacceptably worse in most developing countries due to low efficacy of the vaccines in the region. The vaccines often do not reach the developing countries due to financial and logistic challenges.

A high burden of rotavirus disease and the unresolved challenges with the current rotavirus vaccines, particularly in developing countries, have ignited efforts to develop next-generation low-cost, effective and safe vaccine candidates. Virus-like particles (VLPs) have come into focus for their promising application in vaccination because of their unique and attractive set of properties including safety, self-adjuvanticity, developability, economy and the ability to be formulated for stability without a cold chain. Their impact on human health is already evident through commercialization of VLP vaccines against hepatitis B virus infection, human papillomavirus-induced cervical cancer and hepatitis E virus infection. Rotavirus-like particles (RLPs) with proven preclinical immunogenicity and protective efficacy are considered as safe and effective vaccine candidates. While RLPs comprising multiple viral proteins can be difficult to process, modular VLPs presenting rotavirus antigenic modules are promising approaches in reducing process complexity and cost. Depending on the physicochemical properties, size and /or surface density of modules, modularization may affect production of stable VLP forming subunits, termed capsomeres, and prevent VLP assembly. This thesis demonstrates a multipronged approach for a low-cost production of stable bacterially-produced modular murine polyomavirus capsomeres and *in vitro* assembled VLPs presenting a rotavirus highly conserved peptide epitope (RV10) and a conformational and a virus neutralizing 18 kDa antigen (VP8*), separately. The experimental works in this thesis were carried out to address challenges associated with the adverse effects of inserted module's physicochemical properties, size and density on the production of modular capsomeres and VLPs. Particularly, the expression and purification of stable modular capsomeres for *in vitro* modular VLP assembly and the enhancement of module-specific immune response were investigated. The outcomes of the work in this thesis are: (i) a rapid and simple high-throughput screening method based on dynamic light scattering measurements was developed to identify additives for enhanced stability of modular capsomeres; (ii) using synthetic biology designs, the hydrophobicity of RV10 modules was engineered for enhanced stability of modular capsomeres; (iii) *in vitro* assembled modular VLPs displaying RV10 modules were obtained via a module titration approach using *Escherichia coli* protein co-expression strategy; and (iv) highly stable and

immunogenic modular VLPs displaying conformational VP8* were produced using a combination of *Escherichia coli* protein co-expression, simplified modular capsomere purification steps and cell-free *in vitro* VLP assembly. The outcomes of this thesis expand the potential and plasticity of the murine polyomavirus VLP platform for presentation of antigenic modules regardless of their adverse physicochemical properties and large size. This work introduces, to the best of our knowledge, the first bacterially-produced capsomeres and *in vitro* assembled VLPs displaying either hydrophobic RV10 peptide modules or 18 kDa rotavirus VP8* large antigens. The modular capsomeres (CapVP8*) and modular VLPs (VLP-VP8*) induced high levels of VP8*-specific antibodies in mice. The high immunogenicity of CapVP8* and VLP-VP8*likely indicates protective efficacy and makes them a more viable vaccine candidate for further development to prevent rotavirus in the developing world at affordable cost. The strategies developed in this thesis potentially provides a cost-effective production route for modular capsomere and VLPs presenting other antigenic modules from rotavirus and other target pathogens, highly suitable to manufacturing economics for the developing world.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

The candidate contributed to the following publication and conference during the course of the candidature.

A. Peer-reviewed paper

1. Alemu Tekewe, Natalie K. Connors, Frank Sainsbury, Nani Wibowo, Linda H.L. Lua, Anton P.J. Middelberg. A rapid and simple screening method to identify conditions for enhanced stability of modular vaccine candidates. Biochemical Engineering Journal 2015, 100: 50-58.

2. Alemu Tekewe, Natalie K. Connors, Anton P.J. Middelberg. Design strategies to address the effect of hydrophobic epitope on stability and in vitro assembly of modular virus-like particle. Protein Science 2016, 25: 1507-1516.

3. Alemu Tekewe, Yuanyuan Fan, Emilyn Tan, Anton P.J. Middelberg, Linda H.L. Lua. Integrated molecular and bioprocess engineering for bacterially-produced immunogenic modular virus-like particle vaccine displaying 18 kDa rotavirus antigen. Biotechnology and Bioengineering 2016, DOI: 10.1002/bit. 26068.

B. Conference abstracts

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2. Alemu Tekewe, Natalie K. Connors, Linda H.L. Lua, Anton P.J. Middelberg. Design strategies for production of modular rotavirus vaccine candidates. Australian Institute for Bioengineering and Nanotechnology students` conference. 2 October 2015. Brisbane, Australia.

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1. Alemu Tekewe, Natalie K. Connors, Frank Sainsbury, Nani Wibowo, Linda H.L. Lua, Anton P.J. Middelberg. A rapid and simple screening method to identify conditions for enhanced stability of modular vaccine candidates. Biochemical Engineering Journal 2015, 100: 50-58.

- Incorporated as Chapter 3

Contributors	Statement of contributions
Author Alemu Tekewe (Candidate)	Designed experiments (60%) Conducted experiments (100%) Wrote the paper (60%)
Author Natalie K. Connors	Designed experiments (5%) Wrote the paper (15%)
Author Frank Sainsbury	Designed experiments (7.5%) Wrote the paper (5%)
Author Nani Wibowo	Designed experiments (5%) Wrote the paper (5%)
Author Linda H.L. Lua	Designed experiments (10%) Wrote the paper (5%)
Author Anton P.J. Middelberg	Designed experiments (12.5%) Wrote the paper (10%)

2. Alemu Tekewe, Natalie N. Connors, Anton P.J. Middelberg, Linda H.L. Lua. Design strategies to address the effect of hydrophobic epitope on stability and *in vitro* assembly of modular virus-like particle. Protein Science 2016, 25: 1507-1516.

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Contributors	Statement of contributions
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Author Linda H.L. Lua	Designed experiments (15%) Wrote the paper (20%)

3. Alemu Tekewe, Yuanyuan Fan, Emilyn Tan, Anton P.J. Middelberg, Linda H.L. Lua. Integrated molecular and bioprocess engineering for bacterially-produced immunogenic modular virus-like particle vaccine displaying 18 kDa rotavirus antigen. Biotechnology and Bioengineering 2016, DOI: 10.1002/bit. 26068.

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Author Linda H.L. Lua	Designed experiments (15%) Wrote the paper (20%)

Contribution by others to the thesis

This thesis was drafted and written by the candidate under the supervision of Professor Anton Middelberg and Associate Professor Linda Lua.

Animal handling during *in vivo* immunogenicity test as described in Chapter 5 and Appendix B was performed by the candidate with the help of the technicians from the University of Queensland Biological Resources.

All the data and results presented in this thesis are solely the work of the candidate with exception of the following:

- Chapter 4, Figure 4-5D (i) & (ii) Transmission electron micrographs (TEMs) of virus-like particles (VLPs) were taken by Yuanyuan Fan;
- Chapter 5, Section 5.2.6, Purification of VP8* protein by size exclusion chromatography was performed by Nicolas Pichon;
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Keywords

rotavirus, *Escherichia coli*, murine polyomavirus, vaccine, capsomere, virus-like particles, modularization, high-throughput screening, structural biology, protein co-expression

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List of abbreviations

AF4	Asymmetric flow field-flow fractionation
AH	Alhydrogel®
AIDS	Acquired immunodeficiency syndrome
DLPs	Double layer particles
dsRNA	Double stranded RNA
DLS	Dynamic light scattering
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDIM	Epizootic diarrhoea of infant mice
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
GST	Glutathione-S-transferase
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HTS	High-throughput screening
IgA	Immunoglobulin A
IgG	Immunoglobulin G
INF	Interferon
IPTG	Isopropyl-β-D-thiogalactoside
IRF_3	Interferon regulatory factor 3
ISGs	Interferon stimulated genes
ISREs	Interferon stimulated response elements
LB	Luria Bertani
MCS	Multiple cloning site
MHC	Major histocompatibility complex
MW	Molecular weight
NF-κβ	Nuclear factor-κβ
ORF	Open reading frame
PBS	Phosphate buffered saline
PRRS	Pathogen recognition receptors
RLPs	Rotavirus-like particles
r.m.s	Root-mean-square

RV	Rotavirus
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
TB	Terrific broth
TEM	Transmission electron microscope
TEVp	Tobacco etch virus protease
TX-100	Triton x-100
TW-20	Tween-20
TW-80	Tween-80
UNICEF	United nations children’s emergency fund
USA	United states of America
UV	Ultraviolet
VLP	Virus-like particle
WHO	World health organization

Amino acid	One-letter abbreviation	Three-letter abbreviation
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cyc
Glutamic acid	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

Chapter 1

Project overview

Children under the age of five years are at an increased risk for factors that cause diseases, deaths and disabilities [1,2]. The health of this population age group is a major challenge worldwide because it is highly dependent on provision of good health care and proper nutrition [3], and the use of safe and effective vaccines [4]. There often remain significant disparities in minimizing risk factors and promoting healthy conditions between countries with different levels of socio-economic development [3]. In 2000, the United Nations Millennium Development Goal 4 (MDG4) called for reduction of under-five mortality rate by two-thirds between 1990 and 2015 via eradicating diseases and promoting health [3,5,6]. Although the world has made substantial progress, childhood mortality still remains unacceptably high particularly in developing countries in Africa and South Asia (Fig. 1-1) [5,6]. It is estimated that 5.9 million children died globally before age 5 years in 2015 [6] of which more than 50% died of infectious causes, particularly in low-income developing countries in Africa [5,6].

Diarrhoeal diseases associated with gastrointestinal infections resulting from oral-faecal contamination are the second leading cause of childhood mortality worldwide next to pneumonia. They caused 9% of the total under-five deaths in 2013 [5,6]. Among the various enteric pathogens, rotavirus (RV) was the main causes of diarrhoeal death in children under five in 2013 [7]. It has caused nearly 500,000 deaths worldwide in children under 5 years of age every year [5,8]. The situation is significantly worse in developing countries in sub-Sahara Africa and South-east Asia where more than 80% of the globally recorded deaths in 2008 occurred in the region [8].

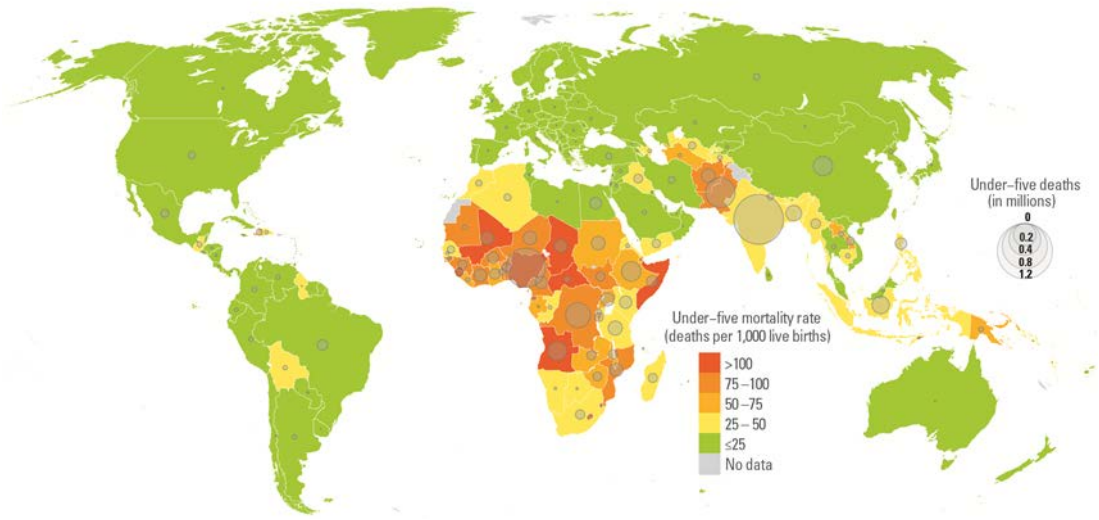

Figure 1-1. Under-five mortality rate and under-five deaths by country. The highest national under-five mortality rates are found in sub-Sahara Africa. The figure was adapted from UNICEF´s *Progress report 2015 [6].*

The treatment option for RV infection is limited to oral rehydration therapy to restore and maintain hydration until the infection resolves [9]. The availability of limited information on therapeutic targets has impeded efforts for RV-specific antiviral drug discovery and development [10]. Investigators in the field have tried to identify possible targets involved in the replication cycle and pathogenesis of different RV strains [10-14]. These targets have been used for *in vitro* and/or *in vivo* screening of different molecules for RV-specific antiviral activity [10,12-14]. Although the results obtained from *in vitro* antiviral screening using cell culture techniques have been encouraging, no chemotherapeutic agent is available to date for treatment of RV infection [11]. Thus, vaccination remains the major and primary prophylactic strategy to reduce the morbidity and mortality from RV diseases [15-17].

The story of RV vaccine development has been a story of starts and stops, of missteps and advances, of great success followed by huge disappointments. RotaShield™ was the first multivalent live-attenuated oral reassortant vaccine against RV. It was introduced into the United States immunization program in 1998 and then withdrawn immediately as it was associated with high risk of intussusception in vaccinated children [15,18]. The experience gained from commercialization of RotaShield™ has broadened understanding of the scientific communities and ignited their efforts to discover and develop safe and effective vaccines. In 2006, two live-attenuated oral RV vaccines, RotaTeq® (Merck) and Rotarix™ (GlaxoSmithKline), were licenced in most developed countries [16]. The introduction of these vaccines has reduced childhood mortality and demonstrated good safety and efficacy profiles in most developed countries [16,18]. However, their efficacy in selected low- and middle-income countries was low during clinical trials

and post-licensure studies [19]. Moreover, the vaccines often could not reach the developing world due to the unresolved financial and logistic challenges [20].

In consideration of these unresolved challenges and the high demand of RV vaccines in the developing world, many investigators are looking for alternative strategies to develop next-generation vaccine candidates to address the difficulties including cost of existing vaccine preparations. These new vaccine candidates can be developed based on rational design and with detailed knowledge of the T-cell and B-cell epitopes [16] and aim to provide an effective weapon against RV. Such vaccines may range from recombinant peptides, recombinant fusion and soluble proteins, and virus-like particles (VLPs). Particularly, VLPs have evolved to become a widely accepted tool for vaccination because of their high safety and efficacy profile [21], their self-adjuvanting property, their ability to present foreign epitopes on their surface, their particulate and multivalent nature [22,23] and their ability to be formulated for stability without a cold chain [24]. Their impact on human health is already evident through the use of marketed VLP vaccines against hepatitis B virus infection, human papillomavirus-induced cervical cancer and hepatitis E virus infection [25,26].

Rotavirus-like particles (RLPs), which are synthetic mimics of the virus, can be a safe and effective alternative vaccine candidate class to marketed live-attenuated oral vaccines [27]. They induced a high immune response and protection in animal studies against challenge with live virus [27,28]. However, a highly complex and expensive bioprocessing of RLPs by recombinant techniques within cells [29] remains one of the major challenges for commercialization of these vaccine candidates for use in the developing world. Therefore, distinct strategies are required to develop safe, effective and cheap next-generation RV vaccines that can reach the developing world.

During the past decade, Professor Anton Middelberg and Dr Linda Lua, and their groups, have developed a low-cost and easily scalable transformational microbial platform technology for manufacturing viral capsomeres and VLPs. The technology uses *Escherichia coli* (*E. coli*) for production of murine polyomavirus VP1 capsomeres that can be assembled into VLPs *in vitro* in cell-free bioreactors [30,31]. VP1 capsomeres and VLPs have also become important platforms for presentation of foreign epitopes in a modular architecture (Fig. 1-2) [32], allowing vaccines to be made in a less complex way for the developing world. Insertion of group A streptococcus J8-peptide epitope at a surface-exposed loop of VP1 has resulted in production of stable modular capsomeres in *E. coli* and *in vitro* assembled modular VLPs [32,33]. Insertion of influenza A M2e-peptide module at the surface-exposed loop or at N- and C-termini of truncated VP1 has also resulted in stable modular capsomeres that do not assemble into VLPs [32,34]. Modularization of

M2e- and J8-peptide modules did not affect the stability of VP1 capsomere and VLP structures. Moreover, the modular capsomeres and VLPs showed clear immunogenicity and protective efficacy in a mouse model [32-34]. However, depending on the physicochemical properties and size of foreign antigenic module, modularization may adversely affect the stability of modular capsomeres, the *in vitro* assembly of modular capsomeres to form modular VLPs and/or the stability of *in vitro* assembled modular VLPs. In this PhD thesis, the development of modular capsomeres and VLPs as novel next-generation RV vaccine candidates was introduced to address a high global burden of RV diseases and the unresolved challenges with the marketed vaccines.

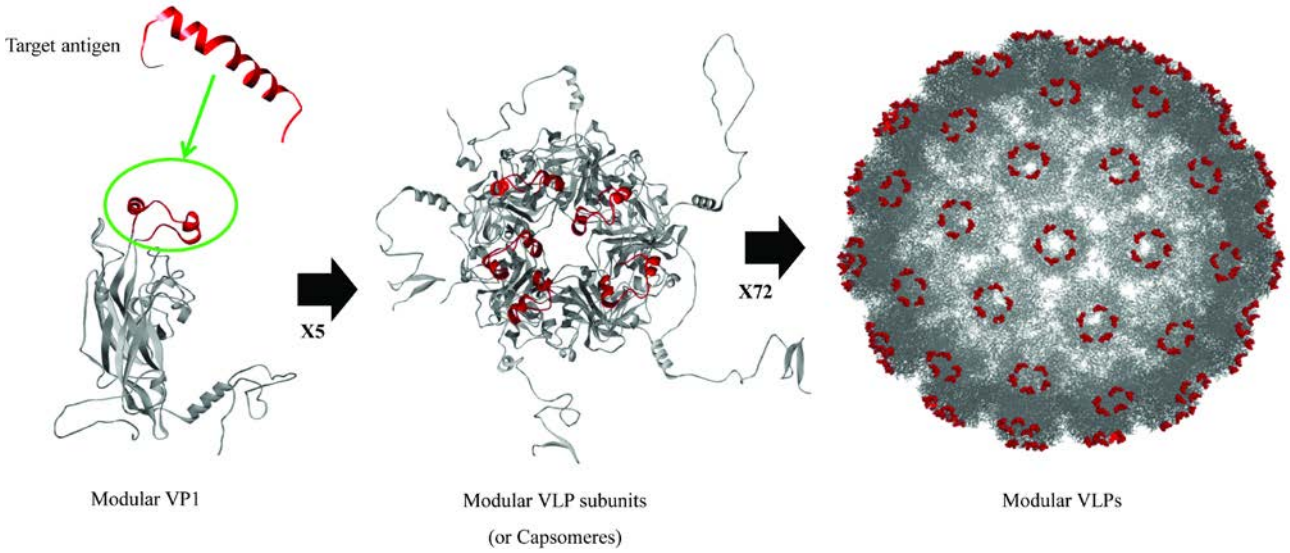

Figure 1-2. Murine polyomavirus VP1 VLP platform for display of foreign antigenic modules in a modular architecture. A relevant target antigen from a target pathogen (red) is inserted at surface exposed engineered insertion site of VP1 at the genetic level. The modular VP1 containing the relevant target antigen is expressed in E. coli as pentameric modular VLP subunits, termed as capsomeres. Then highly purified 72 capsomeres subunits assemble in vitro to form a VLP. The figure was made based on the information available in Middelberg et al.[32].

1.1. Burden of rotavirus

RV is a large and complex virus in the family of *Reovirdae* [35]. It is a non-enveloped and icosahedral viral particle containing 11 segments of double stranded RNAs within its three-layered capsid architecture [36]. The virus shows unusual aspects of structural complexity and has a unique replication cycle. Its triple layer capsid is highly stable for its success as a gastrointestinal tract pathogen and structured properly to protect its genome and deliver it successfully into host cells for viral replication [36,37].

RV strains are classified into seven serogroups (group A-G) based on the genetic variations of the major structural protein VP6 of the virion [38]. Groups A, B and C cause infection in humans. Particularly, RV strains from group A are the most prevalent pathogens associated with diseases in humans, domestic and laboratory animals [39]. These viral strains are the major enteric pathogens causing gastroenteritis and diarrhoea in infants and children under five years of age. They cause about 111 million episodes of gastroenteritis for which health care is not sought and are responsible for 25 million clinic visits, more than 2 million hospital admissions and 500,000 annual deaths in children under 5 years of age worldwide every year [8]. More than 80% of the deaths occur in developing countries. For example, most countries in sub-Saharan Africa have a higher burden of disease and deaths than the rest of the world. There was an estimated 300,000 deaths due to RV diseases in the region in the year 2009 [40]. Children in developing countries of South Asia also bear the greatest burden of deaths primarily due to malnutrition and lack of access to rehydration therapy [41]. A study on the burden of diarrhoeal diseases estimated that RV accounts for close to 40% diarrhoea-related hospitalizations and is responsible for 13% all deaths in children under 5 years of age and kills approximately 150,000 children annually in India [41,42].

RV-related diseases are common in the Middle East and North Africa, which are associated with significant morbidity, mortality and cost [43]. RV is also estimated to cause approximately 40% of all diarrhoea hospitalizations and deaths and a quarter of ambulatory visits for diarrhoea in Latin America and the Caribbean region, resulting in approximately 10 million episodes of diarrhoea annually, two million seeking care in a clinic, 75,000 hospitalizations and 15,000 deaths annually [44,45]. Despite low mortality rate in developed countries of North America, Europe, East Asia and Australia, still there is high incidence of RV disease, imposing a considerable burden upon their health systems and economies [46].

1.2. The current vaccine paradigm

Children under five years of age have experienced one or more RV infections regardless of where they live or their socioeconomic status. The improvements in housing, pure water supply, sanitation, personal hygiene, food quality, nutrition, maternal education and the use of oral rehydration therapy have significantly reduced hospitalizations from bacterial and parasitic diarrheal disease but they are unlikely to reduce the overall incidence of RV infection [46,47]. Consequently, vaccination remains the most effective public health strategy to reduce the global burden of diarrhoeal diseases due to RVs. Vaccine preparations against RVs can generate virus neutralizing antibodies and/or initiate appropriate virus-specific cellular immune responses prior to infection [16,18].

The current rotavirus vaccine paradigm uses two live-attenuated oral vaccines, Rotarix™ (GlaxoSmithKline) and RotaTeq® (Merck), in most developed countries [15-18]. Some developing countries have also incorporated the vaccines in their childhood immunization schedules. The vaccines are effective and significantly reduced the incidence of severe gastroenteritis in infants in high- and middle-income countries [48]. Nevertheless, their efficacy in selected low-income countries, where they are needed most, was low during clinical trials and post-licensure studies [19,49]. The reasons for their lower efficacy in developing countries may be due to high levels of maternal antibodies, mixed infections with various pathogens in the gut, a higher rate of malnutrition, regional differences in prevalent RV genotypes and serotypes, and emergence of potentially virulent strains via reassortment of RVs between vaccine strains and circulating wild type strains [19,47,50]. Their attenuated live nature has raised concerns related to viral shading and risk of reversion and transmission [51]. They have also caused intussusception in some vaccinated infants [52]. The detection of porcine circovirus type 1 DNA in Rotarix™ vaccine and fragments of DNA of porcine circovirus type 1 and type 2 in Rota Teq® as potential contaminants has raised questions with regard to the quality and safety of the vaccines [53]. Moreover, live-attenuated vaccines are often produced using cell culture technology that demands high cost and strict conditions of production, storage, transportation and distribution. This often causes financial and logistic challenges for introducing the vaccines into the developing world [20].

1.3. Next-generation rotavirus vaccines

A high global burden of RV and the unresolved challenges with the marketed live-attenuated oral RV vaccines have ignited efforts to develop next-generation vaccine candidates. The design of the new RV vaccine candidates based on detailed knowledge of T-cell and B-cell epitopes would result in novel new vaccines to address the difficulties of the marketed vaccine preparations (Table 1-1). Such vaccines may range from recombinant peptides, recombinant fusion or soluble proteins [54-56] and RLPs [27]. Particularly, RLPs have gained promising potential as safe and effective alternative vaccine candidates to oral live-attenuated vaccines. They induced a strong immune response and protective efficacy in different animal models against direct challenge with a live virus [27,28].

Table 1-1: Comparison of marketed live-attenuated oral RV vaccines and next-generation VLP-based RV vaccine candidates. The Table was prepared based the information available in [19,20,24,29,32,49,57].

Parameters	***Rotavirus vaccines***		
	Marketed live-attenuated vaccines	*Rotavirus-like particles (RLPs)*	*Modular rotavirus VLPs*
Cost	*Expensive for low-income countries (US$ 2.5-3.5 per dose*	*Expensive due to high manufacturing cost from eukaryotic expression system*	*Affordable for low-income countries (at a calculation cost of less than 1cent per dose)*
Distribution/Logistics	*Challenging and prohibitive for low-income developing countries*	*Easy, particularly, freeze-dried RLPs*	*Easy, particularly, freeze-dried VLPs*
Thermal stability	*Unstable and needs a cold chain system*	*Can be stabilized and do not need a cold chain*	*Can be stabilized and do not need a cold chain*
Efficacy	*Low efficacy in developing countries*	*Showed pre-clinical protective efficacy*	*Pre-clinical and clinical efficacy have not yet tested*
Risk of reversion	*High risk of reversion*	*No risk*	*No risk*
Risk of intussusception	*Risk of intussusception was observed in vaccinated children*	*No risk*	*No risk*

RLPs are non-replicating synthetic mimics of the virus that are made by recombinant techniques within the expression host cells [29,58]. As rotavirus is very complex, the RLPs made inside the cell are also complex, and typically contain unassembled proteins, single layered (assembled VP2), double layered (VLP2/6), and triple layered (VLP2/6/7) RLPs, with or without the VP4 spikes (Fig. 1-3) [29,59]. Production of RLPs inside the cell often involves highly complex, inefficient and expensive bioprocessing steps and suffers from the limitations of yield and scalability [29,58,59]. Simplifying the overall bioprocessing steps, improving efficiency of VLP formation and feasibility for large scale production, and reduction of manufacturing cost have been the focus of many developments to enable the developing world to use the final product with affordable cost.

The ability to manufacture VLPs and their subunits, termed capsomeres, using a transformational microbial viral capsomere and VLP platforms suggests huge potential for cost reduction [57]. Using this technology, the murine polyomavirus capsid protein, VP1, is produced in high yields using a low-cost prokaryotic expression system and scalable chromatographic and non-chromatographic techniques are used for purification of its capsomeres and followed by *in vitro* assembly of capsomeres into VLPs in a cell-free bioreactors [30,31]. The capsomeres and VLPs can also serve

as platforms for display of foreign epitopes in a modular architecture (Fig. 1-2) [32,34]. Insertion of immunologically relevant peptide epitopes or vaccine antigens from influenza A and group A streptococcus to the surface loops of murine polyomavirus capsid protein, VP1, at genetic level through polymerase chain reaction or gene construction have resulted in modular capsomeres and VLPs [32,34,60]. The modular capsomeres and VLPs showed clear immunogenicity and protective efficacy in mice [33,61]. Most importantly, modularization of M2e-and J8-peptide modules can be well tolerated and did not affect the stability and *in vitro* assembly of capsomeres to form VLPs [32,34].

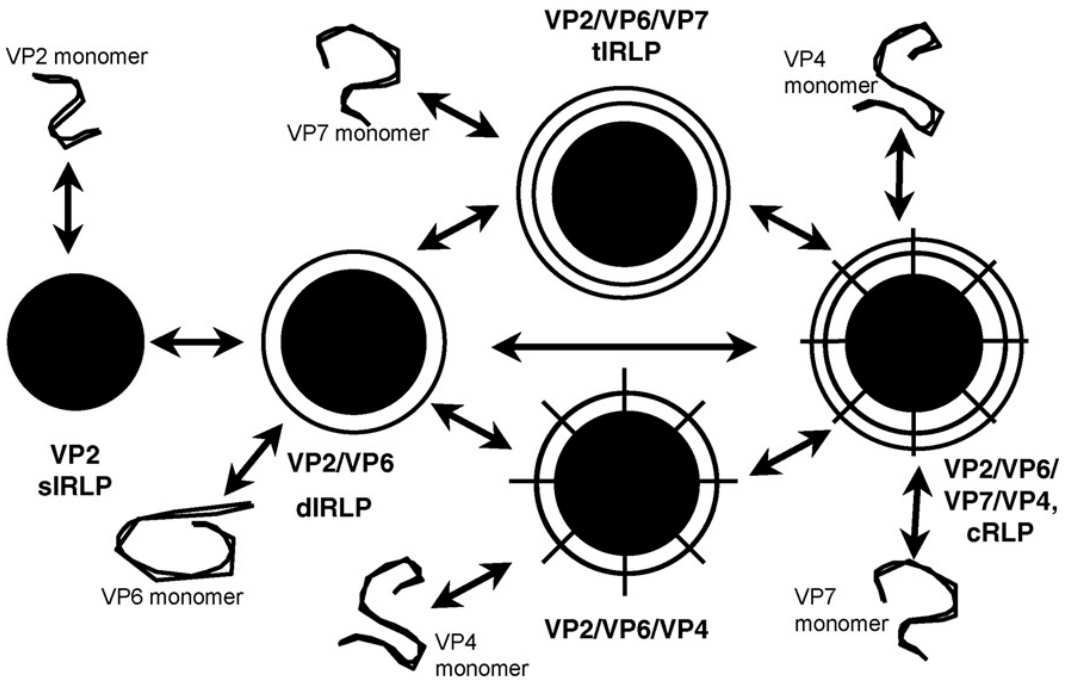

Figure 1-3. Simultaneous expression of four RV structural proteins results in the formation of single-layered RLP (sLRLP), double-layered RLP (dIRLP), triple-layered RLP (tIRLP) and complete RLP (cRLP).The steps followed for assembly of cRPL are unknown. This figure was adapted from Palomares et al.[29].

However, depending on the physicochemical properties and size of modules, modularization may affect the stability and *in vitro* assembly of capsomeres to form VLPs. So far, murine polyomavirus VP1 capsomeres and VLPs have served as a platform for presentation of small (less than 10 kDa) and hydrophilic peptides [32-34,62]. There are some B-cell epitopes, such as the RV peptide epitopes from VP8 subunit domain [63], and many T cell epitopes [64-66], which are hydrophobic peptides. Presentation of these peptides using a capsomere and/or VLP platform may result in modular vaccine candidates that induce protective immunity against RV. It may be also essential to present large antigens in a capsomere and/or VLP platform instead of small peptides to maintain the conformational structure of the immunogenic domains of the antigens for effective binding to B-cell

receptors, antibodies or other arms of the immune system. More recently, Lua *et al*. [60] demonstrated the modularization and presentation of an 18 kDa RV VP8* large antigen on the surface of VP1 VLP, but using a baculovirus-insect cell expression platform for VLP assembly *in vivo*. Thus, the need to use the VP1 capsomere and VLP vaccine platforms for production of next-generation RV vaccine candidates using a low-cost bacterial expression system leads to the general question that this research addresses: are VP1 capsomere and VLP platforms suitable for presentation of hydrophobic peptide epitopes? Can stable modular capsomeres presenting an 18 kDa RV VP8* large antigen be produced using a prokaryotic microbial host for subsequent VLP assembly *in vitro*? If they can be proved to be suitable, the VP1 capsomere and VLP platforms will demonstrate their potential and plasticity for low-cost manufacturing of modular capsomeres and VLPs presenting various heterologous modules regardless of the size and the physicochemical properties of modules.

1.4. Research objectives

The aim of this PhD project is to contribute towards the design and bioprocessing of modular virus-like particles and capsomeres, using *E. coli,* to present a RV cross-reactive and hydrophobic peptide epitope (RV10) and an 18 kDa RV large antigen (VP8*) as next-generation vaccine candidates against RV.

The research covered in this thesis was guided by four main objectives.

(i) to produce in *E. coli* modular capsomeres presenting a RV peptide module and to identify optimum processing conditions for purification of stable modular capsomeres;

(ii) to address the effect of inserting RV hydrophobic peptide modules on protein expression, capsomere stability and in *vitro* VLP assembly using synthetic biology design strategies;

(iii) to develop a simplified bioprocessing route for manufacturing of modular capsomeres and VLPs presenting a RV VP8* large antigen; and

(iv) to assess the immunogenicity of potential modular capsomere and VLP vaccine candidates developed from (ii) and (iii) *in vivo* in mice.

The rationale behind each of these research objectives is explained in the following sections.

1.4.1. Enhancing the stability of modular capsomeres

The stability of modular capsomeres is very crucial, particularly for their application in vaccination, where lower efficacy can be a result of poor vaccine stability [67]. Moreover, production of modular VLPs via *in vitro* assembly in a cell-free reactor often needs highly stable and pure capsomeres, which are stabilized against aggregation. Modularization of capsomeres can be well tolerated [32,34]; however results can be dependent on the physicochemical properties of modules. Insertion of modules may alter protein characteristics. It may also affect the structural features of protein-protein interfaces and the nature of their interactions, and the interaction of amino acid residues and subdomains of proteins with solvent molecules. Alteration of protein characteristics or structural features may lead to formation of insoluble and/or soluble aggregates during downstream processing of modular capsomeres. Thus, depending on the physicochemical properties of modules, modularization may require tailoring of the processing conditions specific to each module to maintain protein colloidal stability.

The nature of the environment surrounding the proteins can be tailored to each protein by adjusting the pH and ionic strength of processing solutions [68,69] or by adding stabilizing additives [69]. Several additives have been studied for their potential to stabilize proteins at different stages of processing, formulation or upon storage. However, identification of the most effective additive from a large experimental space for each target protein is laborious and time-consuming using analytical methods with low-throughput capacity [70]. Therefore, the use of high-throughput screening (HTS) techniques may provide opportunities for simple and rapid screening for the most effective additives. For example, dynamic light scattering (DLS) measurement with high-throughput capacity provides *in situ* analysis within short time periods using small amounts of protein [24]. DLS can be suitable and effective for HTS of protein processing conditions, particularly in cases when the size difference between different species, such as between capsomeres and soluble aggregates, is high.

Due to its high-throughput capacity, simplicity and suitability [24], a DLS-based analysis was used to develop a rapid, simple and effective HTS method to identify optimal processing conditions for enhancing the stability of modular capsomeres (Chapter 3). The developed DLS-based HTS method was able to screen 54 processing conditions in the presence of various additives for their potential to enhance stability of RvVP1 capsomeres. Eight conditions were successfully identified as effective stabilisers, from which the additives, 0.1% (v/v) triton x-100 (TX-100), 0.05% (v/v) TX-100, tween-80 (TW-80) and tween-20 (TW-20) were able to increase stability of RvVP1 modular capsomeres. The potential of these additives in stabilizing RvVP1 capsomeres was further confirmed with high-resolution size exclusion chromatography (SEC) (Chapter 3).

1.4.2. Synthetic biology design strategies for modules

It is clear from Section 1.4.1 that tailoring the nature of the environment surrounding the proteins by adding stabilizing additives can enhance the stability of modular capsomeres. Non-ionic detergents are very mild chemical compounds that do not denature proteins and they increase stability of proteins by inhibiting hydrophobic interactions between proteins or by coating interfaces to modulate adsorption loss and aggregation [71]. However, these additives may be unwanted and have to be removed to use the modular capsomeres for *in vivo* application or to assembly them into modular VLPs *in vitro* in a cell-free reactor. Modular RvVP1 capsomeres did not form modular VLPs when assembled *in vitro* in the presence of non-ionic detergents (Appendix A). Removal of detergents from protein solutions is tedious and not efficient [72]. Their removal often results in re-aggregation of target proteins during further downstream processing or upon storage. Moreover, their efficiency in stabilizing modular capsomeres during downstream processing and the purification yield may depend on the physicochemical properties and size of modules. Particularly, modular capsomeres displaying a hydrophobic peptide module will rarely perform well during upstream as well as downstream processing even under optimized protein expression and processing conditions (Chapter 4). Modularization may also cause structural perturbation of capsomeres or prevent modular VLP formation via *in vitro* assembly from modular capsomeres due to steric hindrance [60].

Synthetic biology has the potential to solve various problems in vaccine development and manufacturing [73]. Low expression level of proteins, poor stability during downstream processing, or low purification yield can be avoided through redesign of the constructs or modules using synthetic biology. Addition of polar and/or charged amino acids into the module sequence would reduce the overall hydrophobicity of the module by providing additional charges or hydrogen bonds and favours expression of soluble and stable proteins in *E. coli*. Insertion of ionic linker sequences into the hydrophobic module sequence can also reduce the hydrophobicity of the peptide module. Linkers showed profound impact on the stability of fusion proteins during expression, processing and storage [74]. They can also separate different moieties of fusion proteins spatially in order to alleviate structural perturbation of moieties, which often compromises the stability and activity of fusion proteins [75]. Previous study demonstrated presentation of an 18 kDa RV large antigen, VP8*, on the surface of VLP using linkers and by reducing the number of antigenic modules on the VLP surface sufficiently below the maximum [60]. The study revealed that longer linkers ensured structural separation and independence between VP8* and the VLP, and a reduction in number of antigenic domains on the VLP surface did avoid a steric hindrance to VLP formation *in vivo* using the insect cell-baculovirus expression system.

In consideration of this successful work in VLP formation *in vivo*, different synthetic biology design strategies were used in this thesis to address the effect of inserting hydrophobic peptide modules on protein expression, stability of modular capsomeres and *in vitro* assembly of modular VLPs (Chapter 4). Insertion of linkers flanking the antigenic module and titration of number of antigenic module on each capsomere via co-expression strategy of unmodified VP1 and modular VP1 in *E. coli* resulted in stable modular capsomeres presenting a RV cross-reactive hydrophobic peptide epitope (RV10) on the murine polyomavirus capsid protein VP1. The modular capsomeres (CapRV10) formed modular VLPs, RV10VLP *in vitro* in a cell-free reactor. RV10VLP is the first bacterially-produced *in vitro* assembled VLPs displaying RV10 (Chapter 4). The developed synthetic biology design strategies enabled the production of CapRV10 and RV10VLP vaccine candidates using a low-cost bacterial co-expression system. The production of RV10-specific antibody against CapRV10 and RV10VLP was also assessed in this thesis. The mice immunized with adjuvanted CapRV10 and non-adjuvanted RV10VLP induced production of comparable ($P = 0.2236$) and low RV10-specific IgG titres ($\sim 10^{2.5}$ and $10^{3.5}$ for CapRV10 and RV10VLP, respectively) (Appendix B).

1.4.3. Molecular and bioprocess engineering for simplified production of modular VLPs presenting large antigens

As mentioned in Section 1.4.2, CapRV10 and RV10VLP induced production of low titres of RV10-specific antibodies. The specific causes for low immunogenicity of CapRV10 and RV10VLP are unknown. It is speculated that induction of high module-specific immune response can necessitate presentation of large conformational antigens on the surface of capsomeres or VLPs. Previous study demonstrated that a modular VLP vaccine containing the protrusion (P) domain of the norovirus capsid protein displaying VP8* antigen has shown anti-VP8* specific immune response and protective efficacy in mice. However, the total expression level of P particle-VP8* chimera is very low in *E. coli* [76,77]. The limitations of purification yield and scalability may also be the challenge of the system as the VLP was made inside an *E. coli* cell, and then purified using low-throughput SEC [76]. More recently, Lua *et al.* [60] has also developed a stable modular murine polyomavirus VLP presenting VP8* using a baculovirus-insect cell co-expression system. Low expression and purification yield, and expensive bioprocessing steps have made the system infeasible for production of VLP-VP8* as vaccines for the developing world.

An alternative and now-proven approach for VLP assembly via cell-free *in vitro* processing has been developed for production of highly immunogenic and protective modular VLPs presenting J8 peptide modules using the murine polyomavirus VP1 VLP vaccine platform [32,33]. Using this

approach, soluble GST-tagged capsomeres presenting small peptide modules are produced in bacteria and purified using chromatographic techniques, and then assembled into VLPs, under engineering control, in a cell-free reactor [31-33]. However, the use of the GST tag adds complexity and cost to the bioprocess due to its multimerization effects, and the demand for affinity chromatography purification and enzyme-mediated tag release steps [78,79]. With the aim to develop safe and cost-effective next-generation vaccine candidates against RV for the developing world, the bioprocess was simplified using module titration strategy for modularization of capsomeres with large VP8* module and expression of non-tagged proteins in *E. coli* for recovery of capsomeres by selective salting-out precipitation that eliminates affinity purification and subsequent enzyme-mediated tag release. It was successfully used for production of stable non GST-tagged capsomeres presenting a RV 18 kDa VP8* module (CapVP8*) with high purity. Modular CapVP8* capsomeres formed modular VLP-VP8* via *in vitro* assembly (Chapter 5). Moreover, the immunogenicity of CapVP8*and VLP-VP8* was assessed in this thesis. The mice immunized with adjuvanted CapVP8* and non-adjuvanted VLP-VP8* developed high (~ 10^5) and comparable (P = 0.6730) VP8*-specific IgG titers (Chapter 5).

1.5. Thesis organization

This PhD thesis consists of 6 chapters, including this introductory chapter.

Chapter 2 provides a literature review of key topics in this research. It includes a review of RV (structure, classification, replication cycle, infection, pathogenesis and disease burden and immunity to rotavirus), live-attenuated oral vaccines, development of next-generation vaccine candidates against rotavirus, strategies for production of modular capsomeres and VLPs by microbial expression system and assessing the immunogenicity of candidate vaccines in *vivo* using animal models.

Chapter 3 focuses on developing a HTS method for rapid identification of buffer additives for enhancing the stability of modular capsomeres [objective (i)].

Synthetic biology design strategies are covered in Chapter 4 [objective (ii)]. The strategies are applied for addressing the effect of inserting hydrophobic antigen sequence on the stability of modular capsomeres and formation of modular VLPs via *in vitro* assembly.

Chapter 5 investigates a combination of molecular and bioprocess engineering approaches for simplified production of bacterially-produced modular capsomeres and *in vitro* assembled VLPs presenting VP8*- a RV large antigen. It also investigates the potential of modular capsomere and

VLP formulations for production of VP8*-specific neutralizing antibodies. *In vivo* immunogenicity tests in mice were carried out to address objectives (iv).

Chapter 6 summarizes conclusions from the work accomplished in this thesis and presents suggestions for future research.

References

1. Hansen C, Paintsil E: **Infectious diseases of poverty in children: a tale of two worlds**. *Paediatric Clinics of North America* 2016, **63**:37-66.
2. Corsi DJ, Mejia-Guevara I, Subramanian SV: **Risk factors for chronic undernutrition among children in India: estimatiing relative importance, population attributable risk and fractions**. *Social Science and Medicine* 2015, **In Press**:DOI: 10.1016.
3. Dedefo M, Oljira L, Assefa N: **Small area clustering of under-five children`s mortality and associated factors using geo-additive Bayesian discrete-time survival model in Kersa HDSS, Ethiopia**. *Spatial and Spatio-temporal Epidemiology* 2016, **16**:43-49.
4. Anderson VL: **Promoting childhood immunizations**. *The Journal for Nurse Practioners* 2015, **11**:1-10.
5. Liu L, Oza S, Hogan D, Perin J, Rudan I, Lawn JE, Cousens S, Mathers C, Black RE: **Global, regional, and national causes of child mortality in 2000–2013, with projections to inform post-2015 priorities: an updated systematic analysis**. *The Lancet* 2015, **385**:430-440.
6. UNICEF: **Committing to child survival: a promise renewed**. *Progress report 2015*:1-100.
7. **Global Burden of Disease Study (GBD) 2013 Mortality and Causes of Death Collaborators: Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the global burden of disease study 2013**. *Lancet* 2015, **385**:117-171.
8. Tate JE, Burton AH, Boschi-Pinto C, Steele AD, Duque J, Parashar UD: **2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis**. *Lancet Infectious Diseases* 2012, **12**:136-141.
9. Basnet S, Mathisen M, Strand TA: **Oral zinc and common childhood infections-an update**. *Journal of Trace Elements in Medicine and Biology* 2015, **31**:163-166.
10. Kim Y, George D, Prior AM, Prasain K, Hao S, Le DD, Hua DH, Chang KO: **Novel triacsin C analogs as potential antivirals against rotavirus infections**. *European Journal of Medicinal Chemistry* 2012, **50**:311-318.
11. Bagchi P, Nandi S, Chattopadhyay S, Bhowmick R, Halder UC, Nayak MK, Kobayashi N, Chawla-Sarkar M: **Identification of common human host genes involved in pathogenesis of different rotavirus strains: an attempt to recognize probable antiviral targets**. *Virus Research* 2012, **169**:144-153.

12. He H, Zhou D, Fan W, Fu X, Zhang J, Shen Z, Li J, Li J, Wu Y: **Cyclophilin A inhibits rotavirus replication by facilitating host interferon type-I production**. *Biochemical and Biophysical Research Communications* 2012, **422**:664-669.

13. Guererero CA, Murillo A, Acosta O: **Inhibition of rotavirus infection in cultured cells by N-acetyl-cysteine, PPARγ agonists and NSAIDs**. *Antiviral Research* 2012, **96**:1-12.

14. Oh HM, Lee SW, Park MH, Kim MH, Ryu YB, Kim MS, Kim HH, Park KH, Lee WS, Park SJ, et al.: **Norkurarinol inhibits toll-like receptor 3 (TLR3)-mediated pro-inflammatory signaling pathway and rotavirus replication**. *Journal of Pharmacological Sciences* 2012, **118**:161-170.

15. Glass R, Parashar UD, Bresse J, Turcios R, Fischer TK, Widdowson MA, Jiang B, Gentsch JR: **Rotavirus vaccines: current prospects and future challenges**. *Lancet* 2006, **368**:323-332.

16. Angel J, Franco MA, Greenberg HB: **Rotavirus vaccines: recent developments and future considerations**. *Nature Reviews Microbiology* 2007, **5**:529-539.

17. Vesikari T: **Rotavirus vaccination: a concise review**. *Clinical Microbiology and Infection* 2012, **18**:57-63.

18. Dennehy PH: **Rotavirus vaccines: an overview**. *Clinical Microbiology Reviews* 2008, **21**:198-208.

19. Mirzayeva R, Steele AD, Parashar UD, Zaman K, Neuzil KM, Nelsong EAS: **Evaluation of rotavirus vaccines in Asia-are there lessons to be learnt?** *Vaccine* 2009, **27**:F120-F129.

20. Madsen LB, Ustrup M, Fischer TK, Bygbjerg IC, Konradsen F: **Reduced price on rotavirus vaccines: enough to facilitate access where most needed?** *Bulletin of the World Health Organization* 2012, **90**:554-556.

21. Kang SM, Kim MC, Compans RW: **Virus-like particles as universal influenza vaccines**. *Expert Reviews Vaccines* 2012, **11**:995-1007.

22. Crisci E, Barcena J, Montoya M: **Virus-like particles: the new frontier of vaccines for animal viral infections**. *Veterinary Immunology and Immunopathology* 2012, **148**:211-225.

23. Kushnir N, Streatfield SJ, Yusibov V: **Virus-like particles as a highly efficient vaccine platform: Diversity of targets and production systems and advances in clinical development**. *Vaccine* 2012, **31**:58-83.

24. Mohr J, Chuan YP, Lua LHL, Middelberg APJ: **Virus-like particle formulation optimization by miniaturized high-throughput screening**. *Methods* 2013, **60**:248-256.

25. Zeltins A: **Constructions and characterizations of virus-like particles: a review**. *Molecular Biotechnology* 2012, **53**:92-107.

26. Lua LHL, Connors NK, Sainsbury F, Chuan YP, Wibowo N, Middelberg APJ: **Bioengineering virus like-particles as vaccines**. *Biotechnology and Bioengineering* 2014, **111**:425-440.

27. Azevedo M, Viasova A, Saif L: **Human rotavirus virus-like particle vaccines evaluated in a neonatal gnotobiotic pig model of human rotavirus disease.** *Expert Review Vaccines* 2013, **12**:169-181.

28. El-Attar L, Oliver SL, Mackie A, Charpilienne A, Poncet D, Cohen J, Bridger JC: **Comparison of the efficacy of rotavirus VLP vaccines to a live homologous rotavirus vaccine in a pig model of rotavirus disease**. *Vaccine* 2009, **27**:3201-3208.

29. Palomares LA, Ramirez OT: **Challenges for the production of virus-like particles in insect cells: the case of rotavirus-like particles**. *Biochemical Engineering Journal* 2009, **45**:158-167.

30. Chuan YP, Lua LHL, Middelberg APJ: **High-level expression of soluble viral structural protein in *Escherichia coli***. *Journal of Biotechnology* 2008, **134**:64-71.

31. Liew MWO, Rajendran A, Middelberg APJ: **Microbial production of virus-like particle vaccine protein at gram-per-litre levels**. *Journal of Biotechnology* 2010, **150**:224-231.

32. Middelberg APJ, Rivera-Hernandez T, Wibowo N, Lua LHL, Fan Y, Magor G, Chang C, Chuan YP, Good MF, Batzloff MR: **A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines**. *Vaccine* 2011, **29**:7154-7162.

33. Rivera-Hernandez T, Hartas J, Wu Y, Chuan YP, Lua LHL, Good M, Batzloff MR, Middelberg APJ: **Self-adjuvanting modular virus-like particles for mucosal vaccination against group A streptococcus (GAS)**. *Vaccine* 2013, **31**:1950-1955.

34. Wibowo N, Chuan YP, Lua LHL, Middelberg APJ: **Modular engineering of a microbially-produced viral capsomere vaccine for influenza**. *Chemical Engineering Science* 2012, **103**:12-20.

35. Arias CF, Isa P, Guerrero CA, Méndez E, Zárate S, López T, Espinosa R, Romero P, López S: **Molecular biology of rotavirus cell entry**. *Archives of Medical Research* 2002, **33**:356-361.

36. Jayaram H, Estes MK, Prasad BVV: **Emerging themes in rotavirus cell entry, genome organization, transcription and replication**. *Virus Research* 2004, **101**:67-81.

37. Greenberg HB, Estes MK: **Rotaviruses: from pathogenesis to vaccination**. *Gastroenterology* 2009, **136**:1939-1951.

38. Matthijnssens J, Otto PH, Ciarlet M, Desselberger U, Ranst MV, Johne R: **VP6-sequence-based cut-off values as a criterion for rotavirus species demarcation**. *Archives of Virology* 2012, **157**:1177-1182.

39. Kirkwood C: **Genetic and antigenic diversity of human rotaviruses: potential impact on vaccination programs**. *The Journal of Infectious Diseases* 2010, **202**:S43-S48.

40. Sanchez-Padilla E, Graise RF, Guerin PJ, Steele AD, Burny M-E, Luquero FJ: **Burden of disease and circulating serotypes of rotavirus infection in sub-Saharan Africa: systematic review and meta-analysis**. *Lancet Infectious Diseases* 2009, **9**:567-576.

41. Jagai JS, Sarkar R, Castronovo D, Kattula D, McEntee J, Ward H, Kang G, Naumova EN: **Seasonality of rotavirus in South Asia: a meta-analysis approach assessing associations with temperature, precipitation, and vegetation index**. *PloS One* 2012, **7**:e38168.

42. Kahn G, Fitzwater S, Tate J, Kang G, Ganguly N, Nair G, Steele D, Arora R, Chawlasarkar M, Parashar U, et al.: **Epidemiology and prospects for prevention of rotavirus diseases in India**. *Indian Pediatrics* 2012, **49**:467-474.

43. Khoury H, Ogilvie I, El Khoury AC, Duan Y, Goetghebeur MM: **Burden of rotavirus gastroenteritis in the Middle Eastern and North African pediatric population**. *BMC Infectious Diseases* 2011, **11**:DOI: 10.1186.

44. Cortes J, Arvelo W, Lopez B, Reyes L, Kerin T, Gautam R, Patel M, Parashar U, Lindblade KA: **Rotavirus disease burden among children less than 5 years of age-Santa Rosa, Guatemala, 2007-2009**. *Tropical Medicine and International Health* 2012, **17**:254-259.

45. Linhares AC, Stupka JA, Ciapponi A, Bardach AE, Glujovsky D, Aruj PK, Mazzoni A, Buendia Rodriguez JA, Rearte A, Lanzieri TM, et al.: **Burden and typing of rotavirus group A in Latin America and the Caribbean: systematic review and meta-analysis**. *Reviews in Medical Virology* 2011, **21**:89-109.

46. Grimwood K, Lambert SB, Milne RJ: **Rotavirus infections and vaccines: burden of illness and potential impact of vaccination**. *Paediatric Drugs* 2010, **12**:235-256.

47. Enweronu-Laryea CC, Sagoe KWC, Glover-Addy H, Asmah RH, Mingle JA, Armah GE: **Prevalence of severe acute rotavirus gastroenteritis and intussusceptions in Ghanaian children under 5 years of age**. *Journal of Infection Developing Countries* 2012, **6**:148-155.

48. Patel M, Steele D, Gentsch J, Wecker J, Glass R, Parashar U: **Real-world impact of rotavirus vaccination**. *Pediatric Infectious Disease* 2011, **30**:S1-5.

49. Patel M, Steele AD, Parashar UD: **Influence of oral polio vaccines on performance of the monovalent and pentavalent rotavirus vaccines**. *Vaccine* 2012, **30**:A30-A35.

50. Wang H, Moon S, Wang Y, Jiang B: **Multiple virus infection alters rotavirus replication and expression of cytokines and toll-like receptors in intestinal epithelial cells**. *Virus Research* 2012, **167**:48-55.

51. Hyser JM, Estes MK: **Rotavirus vaccines and pathogenesis: 2008**. *Current Opinion in Gastroenterology* 2009, **25**:36-43.

52. Desai R, Curns AT, Patel MM, Parashar UD: **Trends in intussusception-associated deaths among US infants from 1979-2007**. *Journal of Pediatrics* 2012, **160**:456-460.

53. Gilliland SM, Forrest L, Carre H, Jenkins A, Berry N, Martin J, Minor P, Schepelmann S: **Investigation of porcine circovirus contamination in human vaccines**. *Biologicals* 2012, **40**:270-277.

54. Ward RL, McNeal MM: **VP6: a candidate rotavirus vaccine**. *Journal of Infectious Diseases* 2010, **202**:S101-S107.

55. Kovacs-Nolan J, Mine Y: **Tandem copies of a human rotavirus VP8 epitope can induce specific neutralizing antibodies in BALB/c mice**. *Biochimica et Biophysica Acta* 2006, **1760**:1884-1893.

56. Xue M, Yu L, Che Y, Lin H, Zeng Y, Fang M, Li T, Ge S, Xia N: **Characterization and protective efficacy in an animal model of a novel truncated rotavirus VP8 subunit parenteral vaccine candidate**. *Vaccine* 2015, **33**:2606-2613.

57. Chuan YP, Wibowo N, Lua LHL, Middelberg APJ: **The economics of virus-like particles and capsomere vaccines**. *Biochemical Engineering Journal* 2014, **90**:255-263.

58. Rodriguez-Limas WA, Tyo KEJ, Nielsen J, Ramirez OT, Palomares LA: **Molecular and process design for rotavirus-like particle production in *Saccharomyces cerevisiae***. *Microbial Cell Factories* 2011, **10**:DOI: 10.1186.

59. Palomares LA, Mena JA, Ramirez OT: **Simeltaneous expression of recombinant proteins in the insect cell-baculovirus system: production of virus-like particles**. *Methods* 2012, **56**:389-395.

60. Lua LHL, Fan Y, Chang C, Connors NK, Middelberg APJ: **Synthetic biology design to display an 18 kDa rotavirus large antigen on a modular virus-like particle** *Vaccine* 2015, **33**:5937-5944.

61. Wibowo N, Hughes FK, Fairmaid EJ, Lua LHL, Brown LE, Middelberg APJ: **Protective efficacy of a bacterially produced modular capsomere presenting M2e from influenza: extending the potential of broadly cross-protecting epitopes**. *Vaccine* 2014, **32**:3651-3655.

62. Neugebauer M, Walders B, Brinkman M, Ruehland C, Schumacher T, Bertling WM, Geuther E, Reiser COA, Reichel C, Strich S, et al.: **Development of a vaccine marker technology: display of B cell epitopes on the surface of recombinant polyomavirus-like pentamers and capsoids induces pepptide-specific antibodies in piglets after vaccination** *Biotechnology Journal* 2006, **1**:1435-1446.

63. Kovacs-Nolan J, Yoo DW, Mine Y: **Fine mapping of sequential neutralization epitopes on the subunit protein VP8 of human rotavirus**. *Biochemical Journal* 2003, **376**:269-275.

64. McNeal MM, Basu M, Bean JA, Clements JD, Choi AHC, Ward RL: **Identification of an immunodominant CD4$^+$ T cell epitope in the VP6 protein of rotavirus following intranasal immunization of BALB/c mice**. *Virology* 2007, **363**:410-418.

65. Choi AHC, Basu M, McNeal MM, Flint J, VanCott JL, Clements JD, Ward RL: **Functional mapping of protective domains and epitopes in the rotavirus VP6 protein**. *Journal of Virology* 2000, **74**:11574-11580.

66. Choi AHC, McNeal MM, Basu M, Bean JA, VanCott JL, Clements JD, Ward RL: **Functional mapping of protective epitopes within the rotavirus VP6 protein in mice belonging to different haplotypes**. *Vaccine* 2003, **21**:761-767.

67. Ajmera A, Scherließ R: **Stabilisation of proteins via mixtures of amino acids during spray drying**. *International Journal of Pharmaceutics* 2014, **463**:98-107.

68. Lee HJ, McAuley A, Schilke KF, McGuire J: **Molecular origins of surfactant-mediated stabilization of protein drugs**. *Advanced Drug Delivery Reviews* 2011, **63**:1160-1171.

69. Matsuoka T, Tomita S, Hamada H, Shiraki K: **Amidated amino acids are prominent additives for preventing heat-induced aggregation of lysozyme**. *Journal of Bioscience and Bioengineering* 2007, **103**:440-443.

70. Gutmann DAP, MizohataI E, Newstead S, Ferrandon S, Henderson PJF, Van Veen HW, Byrne B: **A high-throughput method for membrane protein solubility screening: the ultracentrifugation dispersity sedimentation assay**. *Protein Science* 2007, **16**:1422-1428.

71. Shi L, Sanyal G, Ni A, Volkin DB: **Stabilization of human papillomavirus virus-like particles by non-ionic surfactant**. *Journal of Pharmaceutical Sciences* 2005, **94**:1538-1551.

72. Antharavally BS, Mallia KA, Rosenblatt MM, Salunkhe AM, Rogers JC, Haney P, Haghdoost N: **Efficient removal of detergents from proteins and peptides in a spin column format**. *Analytical Biochemistry* 2011, **416**:39-44.

73. Vohra P, Blakely GW: **Easing the global burden of diarrhoeal diseases: can synthetic biology help?** *Systems and Synthetic Biology* 2013, **7**:73-78.

74. Chen X, Zaro JL, Shen WC: **Fusion protein linkers: property, design and functionality**. *Advanced Drug Delivery Reviews* 2013, **65**:1357-1369.

75. Zhao HL, Yao XQ, Xue C, Wang Y, Xiong XH, Liu ZM: **Increasing the homogeneity, stability and activity of human serum albumin and interferon-α2b fusion protein by linker engineering**. *Protein Expression and Purification* 2008, **61**:73-77.

76. Tan M, Huang P, Xia M, Fang PA, Zhong W, McNeal M, Wei C, Jiang W, Jiang X: **Norovirus P particle, a novel platform for vaccine development and antibody production**. *Journal of Virology* 2011, **85**:753-764.

77. Tan M, Xia M, Huang P, Wang L, Zhong W, McNeal M, Wei C, Jiang X: **Norovirus P particle as a platform for antigen presentation**. *Procedia in Vaccinology* 2011, **4**:19-26.

78. Connors NK, Wu Y, Lua LHL, Middelberg APJ: **Improved fusion tag cleavage strategies in the downstreamprocessing of self-assembling virus-like particle vaccines**. *Food and Bioproducts Processing* 2014, **92**:143-151.

79. Lipin DL, Lua LHL, Middelberg APJ: **Quaternary size distribution of soluble aggregates of glutathione-S-transferase purified viral protein as determined by asymmetrical flow field flow fractionation and dynamic light scattering**. *Journal of Chromatography A* 2008, **1190**:204-214.

Chapter 2

Literature review

2.1. Rotavirus

RV is a non-enveloped and icosahedral virus that belongs to the family *Reoviridae*. RV is a large virus approximately 100 nm in diameter. RV shows unusual aspects of structural complexity for its success as a gastrointestinal pathogen [1,2]. Its genome consists of 11 segments of double stranded RNA (dsRNA), ranging in size approximately from 660 (segment 11) to 3,300 (segment 1) base pairs. The total genome size is approximately 18,550 base pairs. The genome codes for six structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and six non-structural proteins (NSP1-NSP6) and is enclosed by three concentric capsid layers as shown in Fig. 2-1. The inner most capsid layer of RV is formed by 60 dimers of the structural protein, VP2. This layer encloses the genomic RNA and 12 copies of each of two minor structural proteins VP1 and VP3. VP1 is the RNA-dependent RNA polymerase whereas VP3 refers to the viral capping enzymes, guanylyltransferase and methylase [2,3].

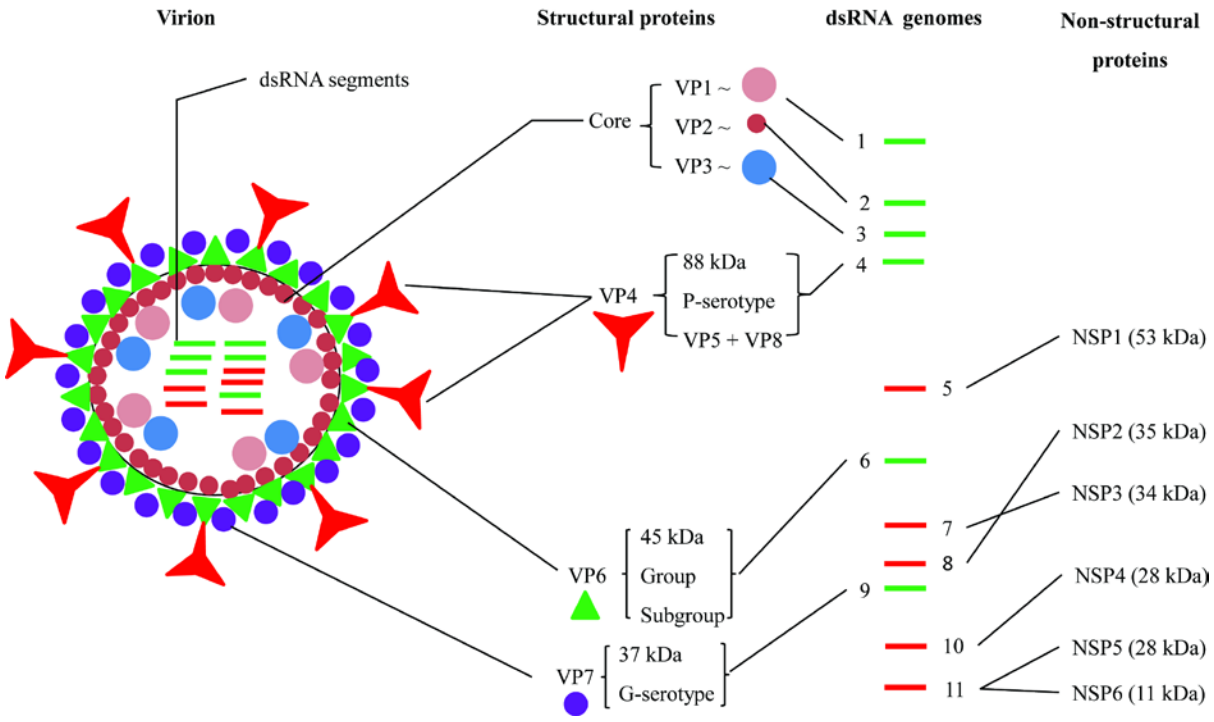

Figure 2-1. The structure of RV virion. This figure was produced based on the information available in Jayaram et al. [4].

The middle layer of the RV capsid shell is formed by 260 trimers of VP6 [3]. VP6 is the most abundant viral structural protein that constitutes approximately 51% of the virion by weight. This protein contains 397 amino acids and has an estimated molecular mass of 45 kDa. VP6 is highly conserved with more than 87% amino acid sequence similarity between any two mammalian group A RV strains [2,3]. It plays key roles in the replication cycle of RV. It is essential for the immature capsid particle to be transcriptionally active and for the budding of the immature capsid particle in to the endoplasmic reticulum where final maturation and assembly of the virus takes place [3,5]. It is also the most immunogenic viral protein as it contains highly conserved and protective T-cell epitopes [5].

The outer most capsid of RV contains two structural proteins, VP7 and VP4. The smooth external surface of the outer capsid is made up of 780 copies of trimers of glycoprotein VP7 (Fig. 2-1) [3]. VP7 is the major constituent of the outer capsid with a molecular weight of 37 kDa in its unreduced and 41 kDa in its reduced form. VP7 is considered as the key mediator of the calcium driven uncoating in RV strains [4]. It is also involved in neutralization of the virus particles [6,7]. It determines the G serotype and contains antigenic domains that are highly conserved within the same serotype but divergent among different serotypes [7]. The minor constituent of the outer capsid shell of RV contains 60 spike-like particles formed by dimers of VP4 (Fig. 2-1). VP4

extends approximately from 12 nm from the VP7 surface [3]. VP4 has a molecular weight of 88 kDa and its cleavage by trypsin in the gut of the host into the C-terminal subunit domain, VP5 (60 kDa) and the N-terminal subunit domain, VP8 (28 kDa) helps the virus to penetrate the enterocytes. VP4 also plays an important role in host cell attachment, haemagglutination, virulence and neutralization [3,4]. VP4 determines P-serotype of the virus and contains cross-reactive epitopes. Particularly, the VP8 subunit domain contains linear B-cell epitopes [8] and large conformational antigenic domains that induce production of virus neutralizing antibodies [9].

2.2. Classification of rotaviruses

RVs are classified into seven serogroups (group A, B, C, D, E, F, and G) on the basis of the antigenic properties or the amino acid sequence variations of the major structural protein VP6 of the virion [10]. There is a vast diversity of genotypes and serotypes within each group. Among the seven serogroups, only groups A, B and C contain viral strains causing infection in humans. Particularly, viral strains from group A are the most prevalent pathogens causing diarrhoea in humans, pigs, cattle, laboratory animals, and birds [11]. Group A RV strains are further classified into two genotypes based on the molecular characterization of the outer capsid proteins, VP7 and VP4. VP7 specifies the G (glycoprotein) genotype whereas VP4 specifies the P (protease-sensitive) genotype [11,12]. G-types are classified as serotypes by neutralization assay or as genotypes by the nucleotide sequencing of VP7. Both the neutralization assay and nucleotide sequencing yield concordant results. Thus, the G-types are referred to by their G serotypes alone or by their G-genotypes alone that are equivalent [1,11]. Unlike G-types, classification of P-types by neutralization and sequencing assays does not always result in concordant results. Consequently, there is often a dual system for P-typing. P serotypes are referred to by their serotype numbers (e.g., P1, P2, P3, etc.) and P genotypes are denoted in brackets (e.g., P [8], P [4] etc.). P genotyping is the most widely used method for classification because of difficulties in standardizing VP4 serotype assay [13]. To date, there are at least 27 G (G1-G27) and 35 P (P [1]-P [35]) genotypes with different combinations of G and P genotypes. For example, more than 70 G-P combinations have been detected from 12 G types and 15 P types identified in humans [14].

Despite the vast potential serotype diversity for human RVs, in nature it does not appear that all G-P combinations are equally efficient in competing for a niche in the human gastrointestinal tract [1]. Globally, human infection with group A RV mainly caused by G1, G2, G3, G4 and G9 in combination with P[4], P[6] and P[8] to form various G-P combinations. Among the different combinations, G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] are responsible for about 90 % of the infections in humans. More importantly, genotype and serotype distribution of group A

RV strains varied between geographical areas [15]. Studies have shown that genotypes G1, G3, G4 and G9 account for 90 % of all RV infections in North America and Europe; however, they are responsible for less than 70% of cases in Africa [16]. Whereas P[8] and P[4] account for over 90% of P types circulating worldwide, their relative frequency seems to be lower in Africa, where P[6] accounts for almost a third of all P types detected in Africa [16,17]. In addition, the genotype and serotype distribution showed temporal fluctuation. Major changes in one or more dominant circulating strain genotypes can occur from one season to another [16].

Besides the molecular characterization of the outer capsid proteins, the genes for expression of less diverse RV proteins have been considered for classification of group A RV strains. To date, the complete open reading frame sequences of all 11 genome segments of almost 80 RV strains have been determined for viruses isolated from human and animal hosts [12]. The Rotavirus Classification Working Group has proposed a classification system based on nucleotide sequence percentage identity cut-off values for each of the 11 group A RV genomic RNA segments. The nucleotide cut-off values are used to distinguish genotypes for each gene segment based on phylogenetic analyses and pairwise sequence identity profiles [12]. This new classification system resulted in the identification of 27G (Glycosylated [VP7]), 35P (Protease-sensitive [VP4]), 16I (Inner capsid [VP6]), 9R (RNA-dependent RNA polymerase [VP1]), 9C (Core protein [VP2]), 8M (Methyltransferase [VP3]), 16A (Interferon antagonist [NSP1]), 9N (NTpase [NSP2]), 12T (Translation enhancer [NSP3]), 14E (Enterotoxin [NSP4]) and 11 H (pHosphoprotein [NSP5/6]) [12,13]. Such a classification system can allow a more complete analysis of unusual RV strains that might emerge because of accumulation of point mutations, genomic reassortment during mixed infections, reassortment between strains of RVs isolated from humans or among strains of human and animal origin, gene rearrangement within gene segments or introduction of animal RV genes into human RV population via direct virus transmission from an animal to a human host [12,14].

The vast genotype diversity of human RV strains across the world and emergence of unusual pathogenic strains due to continuous evolution of RV strains may have important implications for prevention of RV diseases and vaccine development as hetrotypic strains and newly emerging global strains can fail to share neutralizing antigens with the existing vaccine preparations. Thus continuous identification and characterization of globally, nationally or locally prevalent viral strains are very important for the development of vaccines, monitoring vaccine efficacy, and epidemiological study of endemic and epidemic rotavirus diseases.

2.3. Replication cycle of rotavirus

RV is a fascinating virus because of its unusual and complex structure, and its unique replication features [9]. Its highly stable triple-layered capsid is structured properly to protect the RNA genome and to deliver the genetic material into a suitable host cell for its replication and for assembly of the viral particles [4].

The replication of RV takes place in the cytoplasm of matured enterocytes using the host cell metabolic machinery [18]. The matured enterocytes express factors required for virus entry, efficient infection and/or replication. RV attachment and entry into host cells constitute multistep processes. The viral infection involves intestinal protease, two outer capsid proteins, VP4 and VP7, and possible cellular receptors [3,4]. Trypsin-like proteases from the gastrointestinal tract cleave the VP4 (88 kDa) spike protein into VP8 (28 kDa, aa1-247) and VP5 (60 kDa, aa248-776). Trypsin-mediated cleavage of VP4 primes the virus to enter into the host cell [4,18]. RV entry into enterocytes involves interaction of VP8 with sialic acid containing receptors in the initial cell attachment step and interaction of VP5 with integrins such as αvβ3, α4β1, α2β1 during the subsequent post attachment steps [3,4]. The heat shock cognate protein 70, some gangliosides and the cell molecules that are associated with cholesterol and glycosphingolipid-enriched lipid microdomains can also serve as RV receptors [3,19]. Following RV cell attachments, the three-layered virus particle is internalized via non-clathrin- or non-caveolin-mediated endocytosis and traffics to the early endosome (Fig. 2-2), wherein the low calcium concentration is predicted to trigger VP7 disassembly. The dissociation of VP7 trimers mediates virus uncoating, serves as a cue for VP5 rearrangement and allows the virion to penetrate the endosomal membrane [20].

The net result of attachment, uncoating and penetration of the endosomal membrane is the release of the VP2-VP6 double-layer particles (DLPs) into the cytosol of the host cell [18]. The DLPs contain transcriptionally active viral polymerase complexes (VP1 and VP3). The complexes of the polymerases catalyse the synthesis of 11 species of capped, non-poly (A) viral positive single-stranded RNAs ((+) RNAs) using the negative sense RNAs ((-)RNAs) of dsRNA genome segments as templates [18,20]. The nascent (+) RNAs are extruded from a DLP through channels present at each of the 12 vertices of the icosahedral. They serve dual roles during the RV replication cycle, acting as mRNAs for synthesis of viral proteins by cellular ribosomes and as templates for genome replication [18,20,21]. For a typical group A RVs, translation of (+) RNAs gives rise to six structural and six non-structural proteins inside the cytoplasm of the infected host cells (Fig. 2-2).

The non-structural proteins have varied roles in the replication cycle [22]. Some non-structural proteins have only indirect roles in the replication of viral genome or virion assembly. For example, NSP1 functions as an interferon (INF) antagonist to prevent the effect of INF on the expression of viral proteins and production of viral particles [19]. NSP3 is required for the subversion of the host translation machinery [21,22]. The other non-structural proteins, NSP2, NSP5 and NSP4 play direct roles in gene replication and particle assembly [22-24]. NSP2 is a highly basic protein that exists as homo-octamers in the cytoplasm of infected cells [25]. It interacts with another non-structural protein, NSP5, early in the replication cycle. The interaction of NSP2 and NSP5 and their co-localization around transcribing DLPs result in the formation of viroplasms (Fig. 2-2) [25].

The viroplasms correspond to the electron dense inclusion bodies without lipid membranes. They are surrounded by polyribosomes and are localized adjacent to the cell nucleus and near the endoplasmic reticulum. Viroplasms serve as putative sites of viral transcription, genome packaging and replication, and core assembly [18,21,22]. The numerous self- and partner- specific interactions of NSP2 and NSP5 suggest that viroplasms form as large, semi-regular networks designed to sequester viral RNAs and capsid proteins for assembly into nascent virions [20]. Viroplasm-associated (+) RNAs are selectively packaged into assembling VP2 cores. Following encapsidation of the 11 (+)RNAs species, core-associated polymerase complexes perform minus strand synthesis, thereby reconstituting the dsRNA genome inside a pre-virion particle. Then, cores generated within viroplasms interact with VP6 accumulating at the periphery of viroplasms to form DLPs [21-23].

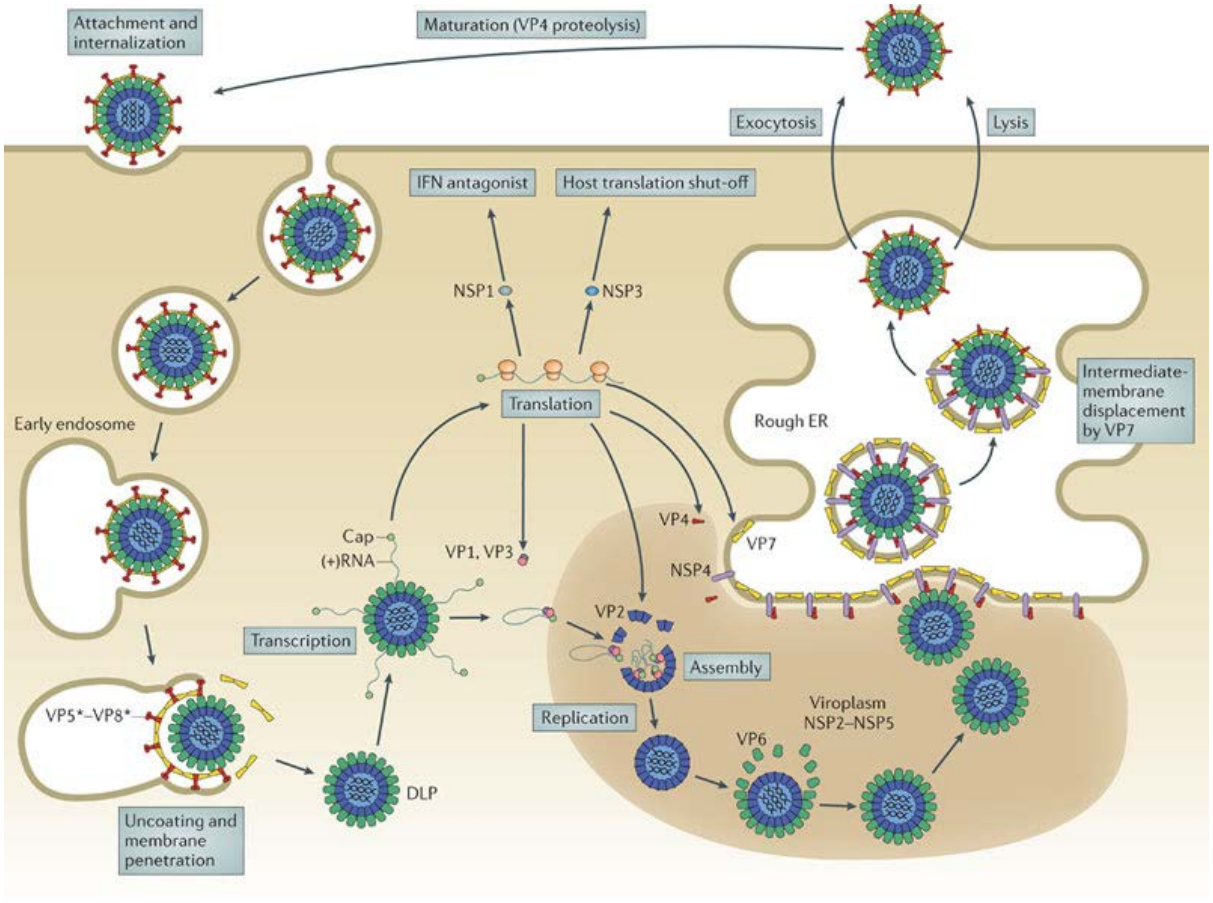

Figure 2-2. The replication cycle of RV. It involves multistep processes. First, the virus enters into the host cell via to cellular receptors and interaction with co-receptors. Following entry, the outer capsid layer, VP7 and VP4, are removed from the particle in the cytoplasm to release the DLPs. The mRNA is produced from DLPs and undergone translation to express 6 structural and 6 non-structural proteins. Assembly of DLPs containing VP2, VP1, VP3 and VP6) and a full complement of 11 ssRNAs is taken place in the viroplasms. Then dsRNAs are synthesised in the precore particles and the particles are mature into triple layered particles by NSP4 acting as an intracellular receptor. Finally, matured particles are released from the cell via exocytosis or lysis and the virus infects other cells to continue its life cycle. The figure was adapted from Trask et al. [20].

Newly made DLPs may amplify the replication cycle by supporting secondary rounds of (+) strand synthesis or may acquire the outer capsid proteins by interacting with the endoplasmic reticulum [21,22]. Budding of DLPs from viroplasms into the endoplasmic reticulum results in transiently-enveloped particles [1,22]. The budding of DLPs into the endoplasmic reticulum is mediated by the affinity of the VP6 for the cytosolic tail of viral endoplasmic reticulum transmembrane protein, NSP4 [22]. The outer capsid proteins, VP4 and VP7, are incorporated into new DLPs during the budding process through protein rearrangements that occur as the transient envelope is lost [1,20]. The high affinity interactions of NSP4 and DLPs, and NSP4 and VP5 are very critical for assembly of VP4 spikes during particle formation in the endoplasmic reticulum before assembly of VP7 Once mature triple-layered particles form, they are predominantly released from cells via either cell

lysis or by a non-lytic trafficking pathway (or exocytosis) (Fig. 2-2) [18,20]. The release of the viral particle from the infected cell exposes the virion to trypsin-like proteases of the gut. Specific trypsin-mediated cleavage of VP4 into VP5 and VP8 produces the fully infectious virion that can infect host cells and the replication cycle continues within the cytoplasm of the host cells as shown in Fig. 2-2 [20].

2.4. Infection, pathogenesis and disease burden

RV is a highly contagious enteric pathogen with low viral doses [26]. The virus is highly stable and able to persist in the environment for long period of time. The presence of RV in the environment carries health risks because of its high stability and its very low infectious viral doses. It is transmitted to susceptible individuals mainly by the faecal-oral route from direct contact with children and adults with subclinical illness or through contact with contaminated hands, fomites, food, water and surfaces [26,27]. The transmission of RVs can also occur from animal to human as well as from animal to animal either by direct contact of the virus or by the contribution of one or more genes to reassortants [28,29]. RV shedding in large quantities from the stools of vaccinated children, or infected individuals and animals can contribute for transmission of the virus between intra- and inter-species of animals and exacerbate the risks of infection [26,29].

RV mainly infects the mature enterocytes in the mid and upper part of the villi of the small intestine [30]. The virus uses its two outer capsid proteins, VP4 and VP7, trypsin-like proteases and cellular receptors [19] to infect mature enterocytes. The spike-protein VP4 has also played critical roles in RV virulence and pathogenesis in different animals and humans [28,30]. In addition, virus virulence is related to the structural protein, VP3 and the non-structural proteins NSP1, NSP2 and NSP4 [28]. Its complex structure with different virulence factors help the virus to cause not only gastrointestinal infections but also systemic infections that may result in clinical consequences in multiple organs [1,28].

An infection of the gastrointestinal tract by RV ultimately leads to diarrhoea without visible organ damage [28,30]. Diarrhoea is the main clinical manifestations of RV infections mainly in infants and young children. It is also sometimes associated with sporadic outbreaks in elderly and immunosuppressed patients [1,31]. RV induced diarrhoea may involve multifactorial pathogenesis mechanisms [32]. One of the pathophysiological mechanisms by which RV induces diarrhoea is malabsorption [1,31,32] due to virus-mediated destruction of absorptive enterocytes, virus-induced down regulation of the expression of absorptive enzymes, and functional changes in tight junctions between enterocytes that lead to paracellular leakage [1]. Local villus ischemia leading to vascular

damage and diarrhoea [31,32], alterations in transepithelial fluid balance [32], reduction in epithelial surface area by replacing the absorptive enterocytes with immature, crypt-like, secretory cells [31,33] and the activation of the enteric nervous system [30,31] are some of the proposed pathogenesis mechanisms of RV induced diarrhoea.

The enterotoxic activity of NSP4 represents another mechanism of RV induced diarrhoea (Fig. 2-3) [31,33,34]. A secreted cleavage product of NSP4 ($NSP4_{aa112-175}$ or the extracellular NSP4) binds to integrins α1β1 or α2β1 on neighbouring cells and triggers the activation of phospholipase C (PLC) that leads to the production of inositol 1,4,5-triphosphate (IP3), IP3 receptor and IP4. The production of IP3 and its receptor mediates calcium release. The calcium activates chloride secretion [33-35]. Calcium mobilization may also trigger the release of different mediators such as cytokines, amines, prostaglandins, peptides and nitric oxide that activate the enteric nervous system, there by stimulating chloride secretion and consequently secretion of water [33,35]. In parallel, osmotic diarrhoea is also induced by several lytic viral cycles [33]. Studies demonstrated that the secretion of chloride is generally age-dependent, occurring in neonatal mice but not in adult mice and leads to diarrhoea [34]. The clinical outcomes of RV infection are affected not only by the viral factors but also by the age of the host. Infants at the age of 3 months to 2 years are highly susceptible to severe RV induced diarrhoea. Although RV can infect adults, severe symptomatic disease is relatively uncommon and can result from infections with unusual virus strain or extremely high dose of virus particularly in case of immunosuppressed individuals [1].

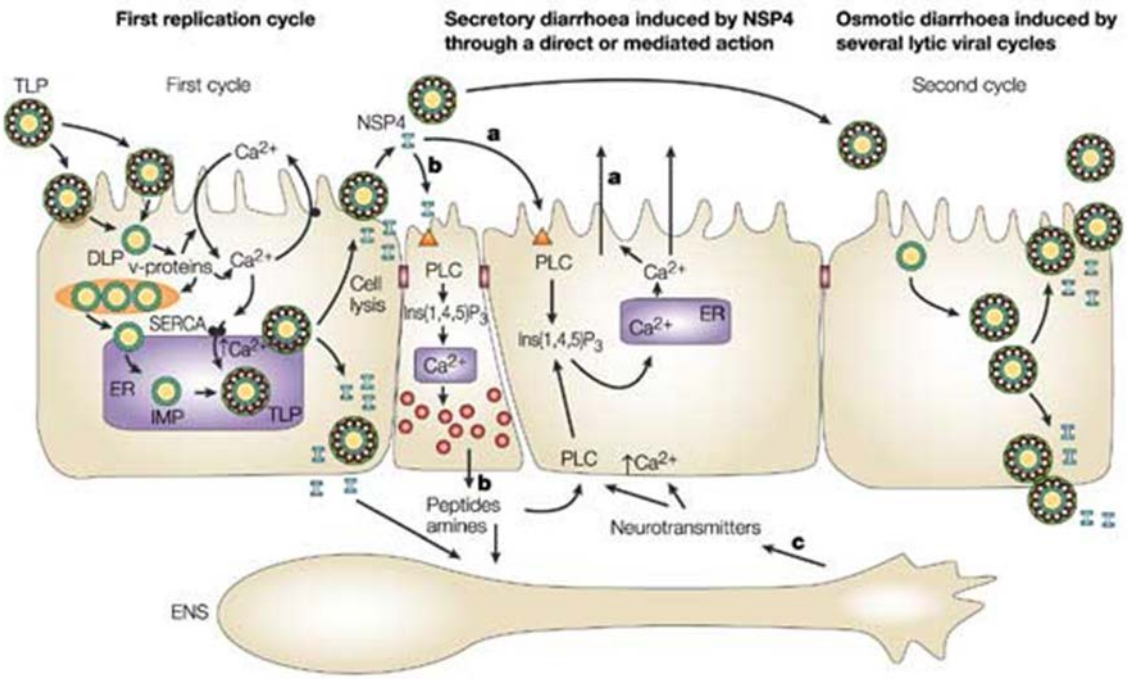

Figure 2-3. Mechanisms for RV induced diarrhoea. Synthesis of viral proteins in the cell cytoplasm during replication leads to an increase in the concentration of Ca^{2+} in the endoplasmic reticulum (ER) that induces activation of Ca^{2+}-dependent enzymes in the cytosol of infected cells. Activated enzymes induce cell lysis and the release of viral proteins and viral progeny. NSP4 might act as a viral enterotoxin on as-yet-uninfected cells to induce secretary diarrhoea through a) Ca^{2+}-dependent secretion by intestinal cells; b) Ca^{2+}-dependent secretion of peptides and amines to stimulate the enteric nervous system (ENS); and c) further activation of epithelial-cell chloride (Cl^{-1}) secretion by the ENS. At the same time, released virus infects downstream absorptive cells that lead to a massive cell death, as a consequence, reduction of the absorptive surface of the intestinal epithelium and an osmotic component of diarrhoea. Various forms of the virus along the RV-maturation pathway are shown: DLP ~ double-layered particles; IMP ~ intramembrane particle; TPL ~ triple-layered particle; SERCA ~ sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase. The figure was adapted from Bomsel et al. [33]

Group A RV strains are the leading causes of gastroenteritis and severe diarrhoea in infants and in young children worldwide [1]. They cause more than 2 million hospital admissions and 500,000 annual deaths worldwide in 2008 in children under 5 years of age More than 80% of the deaths occur in developing countries in Africa, Asia, Latin America and in other developing regions [36]. The disease burden still remains unacceptably worse in most developing countries [37].

2.5. Immunity to rotavirus

Multiple RV infections occur in children throughout their childhood. Each natural infection can result in the development of protective immunity against severe RV induced acute gastroenteritis and recurrent gastroenteritis [38]. The innate immunity to RV infection is acquired after early exposure and acting as the first line of defence against subsequent viral infection[1,39]. It uses

pathogen recognition receptors (PRRs) as primary sensors for viral infection to confer protection against subsequent severe disease through rapid production of cytokines [40]. The interaction of RV dsRNA with PRRs during replication leads to the activation of IFN regulatory factor 3 (IRF3) and nuclear factor (NF)-κβ. IRF3 and NF-κβ translocate from the cytoplasm to the nucleus and stimulate transcription of interferon stimulated genes (ISGs) and type I IFN [41]. Type I IFNs, particularly IFN-α and IFN-β subtypes, are critical for an effective innate immune response against RV [42].

INF-α4 and INF-β are also secreted and bound to their respective IFN receptors to activate the dsRNA-dependent kinases, Jak1 and Tyk2 [38]. The activated kinases phosphorylate and activate transcription factors STAT1 and STAT2 to form a heterotrimeric transcription factor complex (ISGF3) with a third transcription factor, IRF9. ISGF3 translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) to induce transcription of hundreds of ISGs. INF and ISG transcripts can play a critical role in the innate immune response and interferes with the replication of the virus at various stages in the replication cycle [38,42,43]. The role of the non-specific innate immunity in modulating RV infection *in vitro* and in animal models has been known, but the role in human beings is not well explored [1]. This wing of the immune system based on type I IFN response may not be always efficient in providing protective immunity against RV infections. The virus often uses its NSP1 to escape from the type I IFN-mediated immune response [43]. In addition, the innate immunity does not prevent the host from reinfection [38].

The adaptive immune response can be another important wing of the immune system in resolution of ongoing RV infection and protection against subsequent infection [44]. It usually appears later and protects against reinfection or severe diarrhoea in young children or animals when they are reinfected [45]. Some studies in experimental animals and humans suggested that the virus-specific antibody- and cell-mediated immune responses are elicited by natural RV infection or RV vaccination [44]. The exact immunological mechanism by which the adaptive immunity provides protection against RV after natural infection or after immunization is still incompletely understood [46]. The mechanisms of the immune responses to RV are very complex and vary depending on the animal species studied [39].

There has been great interest in identifying the correlates of protection using animal models and humans after both natural infection and vaccination. The production of both systemic and mucosal RV-specific antibodies plays an important role in protection against reinfection. Neutralizing antibody to VP4 and VP7 can block enterocyte infection directly when present in the intestine (Fig. 2-4) [39,46]. However, full correlation of anti-VP4 and anti-VP7 neutralizing antibodies with

protection is still unclear in both animals and humans following natural RV infection and RV vaccination [47]. Correlates of protection after vaccination often vary depending on the type of immunogen and vaccines. In case of heterologous vaccines, serum antibodies may not provide adequate correlates of protection whereas serum IgA may reflect antiviral activity in case of homologous vaccines [46].

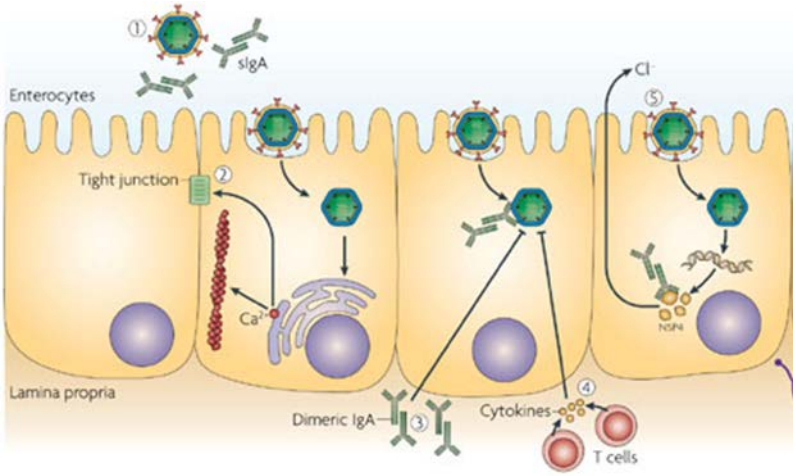

Figure 2-4. Potential mechanisms of the adaptive immunity to RV. In step 1, neutralizing antibodies, mainly secretary IgA (sIgA), directed against the outer capsid proteins can prevent viral binding and penetration, inducing viral exclusion. If the mechanism in step 1 fails the virus will infect the cell and replication will take place in step 2. Viral replication can be inhibited by secretary anti-VP6 dimeric IgA during transcytosis across enterocytes as shown in step 3. In step 4, cytokine-secreting RV-specific T-cells can also inhibit virus replication. If viral replication is not stopped in step 4, replicating virus produces NSP4. Antibodies against NSP4 could potentially prevent diarrhoea, which will be induced by direct or mediated action of NSP4. This figure was adapted with slight modification from Angel et al. [39].

Besides the neutralizing antibodies against VP4 and VP7, some studies revealed the protective immunity of antibodies against VP6 and NSP4 proteins (Fig. 4) [39,48]. Studies using different animal models demonstrated induction of VP6-specific antibody of the IgA class [49]. The VP6-specfic IgAs in the intestine are able to clear RV infections via non-neutralizing or intracellular neutralizing immune responses following transcytosis via enterocytes into the gut lumen [5]. Studies have also investigated the protective role of antibody produced against NSP4 and $NSP4_{aa114-135}$ peptide [47,48]. Antibody to NSP4 can block diarrhoea via its anti-enterotoxin activity, but cannot prevent viral infection [50]. For example, mouse dams fed transgenic potato tuber expressing cholera toxin-murine RV $NSP4_{aa114-135}$ peptide fusion protein were found to develop NSP4-specific serum and intestinal antibodies. The mouse pups born to such dams were partially protected by passively acquired anti-NSP4 antibodies against diarrhoea induced by simian RV SA11 [31,48].

The cell-mediated immune arms of the adaptive immunity may also play a role in protection of RV infections. Experiments in animal models have demonstrated an important role of cell mediated immunity in protection from RV shedding [51]. The adoptive transfer of both immune splenic and intestinal $CD8^+$ T cells, and splenic $CD4^+$ T cells has shown clearance of chronic RV infection using the mouse model [52]. Particularly, different studies have investigated the critical role of $CD4^+$ T cells in VP6-induced protection of mice against RV shedding [52,53]. Studies using the murine model suggested that T cells can play an important role in antiviral immunity since $CD4^+$ T cells contribute to the development of RV-specific IgA by memory B cells and RV specific $CD8^+$ T cells accelerate the resolution of primary virus infection [53]. Some studies using animal models also demonstrated the role of the cell-mediated immune response in resolution of primary RV infection and in providing protective immunity against RV re-infection through production of cytokines by activated T cells [52,54]. Particularly, the Th1 cytokines, such as IFN-γ, play a major direct and/or indirect role in the defence against RV infection [55].

Although the role of cell mediated immune response in humans for protection from RV infection or vaccination is not well characterized [52], results from different studies using animal models suggested that the cell mediated immune response with its various components is an important wing of the adaptive immunity in resolution of primary RV infection and in providing protective immunity against RV re-infection.

2.6. Strategies for control and prevention of rotavirus

Virtually all children will have experienced RV infection during their childhood independent of the socioeconomic status the countries where they live. RV gastroenteritis complicated by dehydration remains a major cause of child mortality, morbidity and hospitalization [37]. Hygiene and sanitation practices are not sufficient to prevent the spread of RV infection within the community and to reduce the burden of the diseases at regional, national and/or global level. The treatment option for RV is primarily supportive and consists of rehydration and restoration of electrolyte balance until the infection resolves [56]. However, coverage levels of rehydration therapy remain low particularly in most developing countries due to inadequate manufacturing capacity and lack of access to good health care delivery facilities and infrastructure [57]. Clinical trials have also demonstrated the effectiveness of some probiotic preparations in reducing the severity and duration of RV-induced diarrhoea in children and adults [58,59]. In addition, some studies have been carried out to identify possible therapeutic targets that are involved in the replication cycle and pathogenesis of RVs [60-62]. Availability of limited information on the therapeutic targets has impeded the discovery and development of RV-specific antivirals [62]. More recently, some of the targets have been used for

in vitro and/or *in vivo* screening of different molecules for RV-specific antiviral activity. Although the findings from RV-specific *in vitro* antiviral screening using cell culture techniques have been encouraging [61-65], no RV-specific chemotherapeutic agents are available to date for clinical use [60]. Thus vaccination remains to be the major and primary prophylactic strategy to reduce the morbidity and mortality from RV diseases [39]. Vaccine preparations against RVs can generate virus neutralizing antibodies and/or initiate appropriate virus-specific cellular immune responses prior to infection [39,66]. The current vaccine paradigm consists of live-attenuated oral RV vaccines. In addition, different non-replicating subunit vaccine candidates against RV are under development.

2.6.1. Live-attenuated vaccines

A live-attenuated tissue-culture-grown RV strains formulated for oral delivery would best mimic the natural immunity induced by wild-type RV infection in the intestines [39,66,67]. So far different live-attenuated RV oral vaccines have been developed and tried/or used to prevent the burden of RV. Bovine RV (RIT 4237) was the first candidate live oral vaccine. This vaccine candidate was derived from a bovine strain Nebraska calf diarrhoea virus, P6[1]G6, and named RIT 4237 for use in human trials. Its cross-protective efficacy against human RV strains in studies in gnotobiotic pigs initiated human trials [68]. A series of human trials were conducted in Finland from 1982 to 1986, starting from phase I studies in adults and rapidly progressing to efficacy trials in infants. However, the RIT 4237 vaccine failed to show consistent efficacy in trials in developing countries and its further development was soon abandoned [67,68].

Rhesus RV was introduced as an alternative heterologous RV vaccine candidate to bovine derived RIT 4237 [69]. This vaccine candidate multiplied more efficiently in human intestines and showed greater immunogenicity compared with RIT 4237. However, Rhesus RV alone did not show impressive protective efficacy in Finland and in the USA [67]. Moreover, its protective efficacy was likewise inconsistent [69].

The development of reassortant strains having the attenuation properties of the animal strains and individual genes encoding the outer capsid proteins of common human RV strains improved the efficacy of monovalent vaccines [66,70]. Reassortment of the Rhesus RV vaccine backbone with human RV VP7 proteins representing the G-types G1, G2 and G4 resulted in the human-rhesus RV tetravalent vaccine [66,69]. This vaccine candidate showed higher efficacy against severe RV gastroenteritis in USA and in Finland and, it was also quite efficacious in Venezuela [67]. It was the first multivalent live-attenuated oral reassortant vaccine licensed as Rotashield^{TM} and

incorporated into the USA infant immunization program in 1998 against RV [69]. But its association with high risk of intussusception in vaccinated children led its withdrawal from the program a year after its introduction [66,70].

In 2006, WHO recommended two live-attenuated oral RV vaccines, Rotarix™ and RotaTeq® for use in the USA, Australia, many European and Latin American countries where evidence of efficacy was demonstrated [39]. The human monovalent RV vaccine was originally developed by isolating the viral strain (strain 89-12) infecting a child in Cincinnati and by tissue culture passage of a wild type strain using African Green monkey kidney cells [39,66]. This vaccine is a P1A[8]G1 strain thus represents the most common of the human RV VP7 and VP4 antigens [66,70]. The vaccine was prepared based on the rationale that a vaccine containing a human RV G1P[8] strain can induce serotype-specific immunity against the most common human G-type and P-type RVs [67,70]. It was further developed by Avant Immunotherapeutics and licensed to GlaxoSmithKline Biologicals. GlaxoSmithKline Biologicals further modified the vaccine by cloning and tissue culture passaging of the parent vaccine strain [66,69]. The resulting vaccine, RIX4414 (Rotarix™) (Fig. 2-5), underwent initial trials in Finland and showed safety, immunogenicity and efficacy [66]. Clinical trials conducted across Europe, Latin America, Asia with this vaccine also demonstrated high efficacy against severe gastroenteritis caused by circulating wild-type RV. Generally, Rotarix™ was highly efficacious in preventing severe gastroenteritis in infancy in high and middle income countries [39,66,67,70].

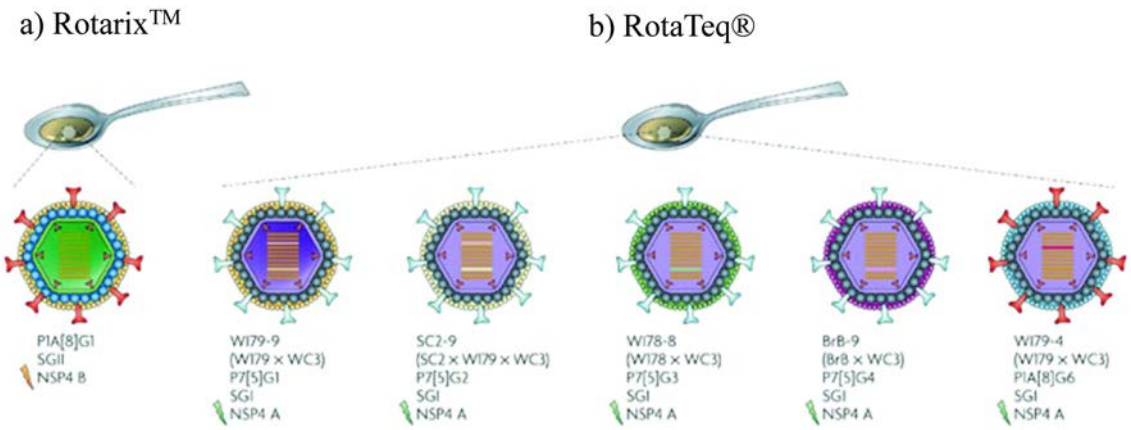

Figure 2-5. Marketed live-attenuated oral RV vaccines. (a) Rotarix™ is a monovalent an attenuated human RV vaccine made of a tissue-culture-adapted human P1A[8]G1, VP6 subgroup II and NSP4 geno-group B strain. (b) RotaTeq® is a bovine (WC3)-human reassortant vaccine composed of the five strains, each containing a human RV gene encoding the VP7 neutralizing protein from different serotypes. Notably, in the WI79-9 and SC2-9 viruses (the last was used to create the first), genes 3 (VP3) and 9 (VP7) are of human origin. The figure was adapted from Angel et al.[39].

The bovine-human pentavalent reassortant RV vaccine [RotaTeq® (Merck and Co., Whitehouse station, New Jersey, USA)] is an oral, pentavalent, live-attenuated human-bovine mono-reassortant vaccine (Fig. 5) [39]. The backbone of RotaTeq® is the bovine RV strain WC3, which is a low passage level isolate of calf RV that was used as a candidate vaccine in the 1980s [67]. The WC3 strain (G6P7[5]) was reasserted with VP7 surface proteins of human RVs G1, G2, G3 and G4, and human VP4 type P1A[8] RV to develop the pentavalent vaccine [67,69]. RotaTeq® was licensed in the USA, Australia, and many European and Latin American countries since 2006. It was safe, immunogenic and highly efficacious against RV during clinical trials and significantly reduced the incidence of severe gastroenteritis in infants in developed and middle income countries [39].

Both Rotarix™ and RotaTeq® have shown good safe and efficacy profiles in preventing severe diarrhoea among children in middle- and high-income countries [39,66]. However, their efficacy in selected low-income countries of Africa, Asia and Central America, where they are needed most, was low during clinical trials and post-licensure studies [71,72]. The restricted immunogenicity and efficacy of the vaccines in developing regions may be due to host-related aspects, socio-cultural and socioeconomic differences, vaccine related characteristics, the higher prevalence of enteric infections, presence of other co-morbidities such as HIV/AIDS, tuberculosis, malaria, concomitant administration of other oral viral vaccines [71], regional differences in prevalent genotypes and serotypes, and reassortment between vaccine strains and circulating wild type strains or among vaccine strains [11]. Moreover, potential safety issues with the vaccines are yet to be clarified; the

mechanisms by which they induce protection and the molecular basis of their attenuation are not well understood [39]. Their live-attenuated nature raised some concerns related to viral shedding, reversion to virulent strains and the risk of transmission and intussusception [73]. The detection of porcine circovirus type 1 DNA in Rotarix™ vaccine and fragments of DNA of porcine circovirus type 1 and type 2 in Rota Teq® as potential contaminants has also raised questions with regard to the safety and quality of the vaccines [74]. In addition to these challenges, financial and logistic obstacles are also limiting the use of the vaccines in developing countries [75]. Because of high cost and low efficacy of Rotarix™ and RotaTeq®, only few developing countries with the highest RV-related mortality rates have introduced the vaccines into their routine Expanded Program for Immunization schedules with the help of donating organizations [76].

Another live-attenuated oral rotavirus vaccine, the Lanzhou Lamb RV vaccine, has been licensed in China since 2000. This vaccine was developed directly from an ovine animal strain, group A (G10 P[12]) [77,78]. Administration of one dose of this vaccine conferred partial protection in children [78]. Several other live-attenuated RV vaccine candidates are under development: the bovine (UK strain)-human reassortant vaccine, the human neonatal RV-3 strain, and the natural bovine-human reassortant neonatal 116E strain have progressed to different stages [69,77]. The bovine RV pentavalent vaccine contains RV human-bovine (UK) reassortant strains of serotype G1, G2, G3, G4 and G9. It has been developed by the serum Institute of India Ltd, in collaboration with the National Institute of Allergy and Infectious Diseases, USA. This vaccine candidate was safe and immunogenic during toxicity studies in animals and, Phase I and II clinical studies in adults, children and infants [79]. The 116E RV strain, which has an unusual G9P[11] genotype, is a naturally bovine-human reassortant strain containing one 10 gene segments from a human RV, and a single gene encoding spike protein from a bovine strain [80,81]. This live-attenuated vaccine candidate was developed recently as part of the Indo-US vaccine action program [81]. Studies showed that 116E RV vaccine against severe RV gastroenteritis and severe gastroenteritis of any cause was efficacious in Indian infants [82]. However, no complete data for efficacy in the second year of life and no results from post-marketing surveillance study available to date. The 116E vaccine caused intussusception in few Indian infants during studies for efficacy and safety [81,82]. Appaiahgari *et al*. [80] also demonstrated the interference of maternal antibody on the immunogenicity of oral RV 116E vaccine in Indian infants.

2.6.2. Inactivated vaccines

Observable progress has been achieved in the last four decades in the accelerated development of live-attenuated oral RV vaccines. However, improved vaccines are still needed, particularly in

developing countries where the burden of the disease is the highest but where the currently used oral vaccines have been the least effective and costly and logistically fragile [75,76]. The use of inactivated RV vaccines have been suggested as an alternative to live-attenuated oral vaccines since they pose several scientific and administrative advantages to the live-attenuated ones [83]. They have greater efficacy for children in developing world and do not cause intussusception and other adverse events [83,84]. In different animal models, partial or complete protection has been induced using parenteral immunization with inactivated RV. For example, Johansen *et al.* [85] have demonstrated that formalin-inactivated RV given intramuscularly with the adjuvants, MPL® or L3®, stimulated a potent immune response with high neutralizing antibody titres and induced protective immunity in infant mouse model. Wang *et al.* [84] demonstrated that a very low dose of thermally inactivated RV formulated with aluminium hydroxide was highly immunogenic in mice. However, they got significantly lower immunogenicity and less protective immunity of the inactivated human strain CDC-9(G1P[8]) vaccine formulated with aluminium phosphate against RV infection in gnotobiotic piglets [84]. Inactivated RV vaccine candidates are also potentially less costly and quicker to develop since, in the absence of concern for intussusception, the vaccine can be tested in clinical trials with fewer than 10,000 infants at a substantially lower cost. However, inactivated RV vaccines could not provide endogenously synthesized proteins and generally do not elicit cytotoxic T cell responses that may be important in resolution of RV infections [83]. In addition, they often need toxic adjuvants for boosting their protective immune response [85].

2.6.3. Subunit vaccines

Subunit vaccines are non-living and containing clearly defined and carefully controlled antigens or antigenic subunit domains. They pose no risk of reversion to a virulent state and antigen competition typical of complex live vaccines can be minimized when subunit vaccines are used [86]. The subunit vaccines against RV may range from recombinant peptides, recombinant fusion and soluble proteins, RLPs and chimeric VLPs.

2.6.3.1. Recombinant peptides and proteins

Several studies demonstrated the immunogenicity of some recombinant RV structural and non-structural proteins and recombinant peptides derived from RV proteins in different animals, in naturally-infected children and adults, and in vaccinated children [5,48,87]. The major proteins with high potential for vaccine development are VP6, NSP4, VP7 and VP4 [66].

VP6 is the most immunogenic and conserved RV protein [5]. It contains several highly conserved group-reactive epitopes. A study of VP6 epitopes has been conducted using pools of synthetic

overlapping peptides representing the entire antigen sequence, particularly those peptides overlapping with the amino acid sequences of the carboxyl terminal half of the VP6 protein of the murine RV strain [88,89] and human RV G1P[8]A strain [90,91]. A highly conserved and protective $CD4^+$ T cell epitopes were identified from VP6 protein of porcine RV YM, simian RV SA11 strains [92], murine RV (Epizootic Diarrhoea of Infant Mice (EDIM)) strain [88,89,93], G1P[8] human RV infected rhesus macaque [90]. Major histocompatibility complex (MHC)-class I-restricted $CD8^+$ T cell-binding epitopes were also identified from RV VP6 protein [89,91,94]. Some of these $CD8^+$ T cell epitopes were partially protective in $H\text{-}2^b$ C57B1/6 mice model [89,94].

The identification of highly conserved and protective or partially protective T-cell epitopes from VP6 has ignited efforts to develop subunit vaccines containing VP6 and/or peptides derived from VP6. Several promising candidate VP6-based subunit vaccines have been produced from *E. coli* [95-97], transgenic plants [98,99] and the milk of transgenic rabbits [100]. Intranasal administration of *E. coli*-expressed chimeric VP6 protein along with the mutant *E. coli* heat-labile enterotoxin [LT(R192G)] adjuvant has consistently resulted in approximately 99% reductions in RV shedding after subsequent EDIM challenge [95-97]. Passively immunized mouse pups born from dams immunized with plant-expressed chimeric VP6 exhibited reduced diarrheal symptoms compared with the non-immunized ones following challenge with virulent RV strains [98,99]. Studies revealed that vaccines-based on VP6 protein generated RV specific faecal secretory IgA, systemic IgG and IgA and a RV-specific Th1 response [94,100,101]. Non-neutralizing anti-VP6 antibodies are highly cross-reactive among all group A RVs and inducing heterotypic protection [96,98,99]. Anti-VP6 IgA was able to confer protection by inhibiting viral transcription at the start of the intracellular phase of the viral replication cycle [98,99,102]. Moreover, immunization with VP6 might prime the immune system for enhanced production of neutralizing antibodies against the outer capsid proteins VP4 and VP7 upon challenge with homotypic or heterotypic viruses [98,99]. A study also demonstrated that the protection elicited in neonatal mice after VP6/LT(R192G) immunization was associated with T cell responses, particularly those cells that were stimulated to produce Th1- and ThIL-17 specific cytokines [103].

NSP4 is another highly conserved protein with high potential for vaccine development against group A RVs infecting mammalian species [104]. Hyser *et al.* [105] identified four B-cell epitopes from the RV SA11 NSP4 cytoplasmic tail by use of monoclonal antibodies and epitope-specific antibodies. All of these epitopes were considered to be linear epitopes as the antibodies were able to detect denatured wild-type NSP4. Among these epitopes, $NSP4_{aa114\text{-}135}$ is highly conserved and two of the critical residues E120 and E122 are 100% conserved in all reported sequences [105].

Moreover, previous study on immunization with the synthetic peptide, $NSP4_{aa114-135}$ demonstrated the generation of protective antibody against RV induced diarrhoea [34].

NSP4 is the first described viral enterotoxin [31]. It induces dose-and age-dependent diarrhoea in neonatal mice without causing histological alterations [32,35,106]. An antibody directed against NSP4 may protect the host from NSP4- and RV-induced diarrhoea like that of antibody responses directed solely against the secreted bacterial enterotoxins protect the host against many of the bacterial infections [34]. Studies have demonstrated that both humoral and cellular immune responses stimulated against a recombinant NSP4 or a synthetic $NSP4_{aa114-135}$ peptide in different animals, in naturally infected children and adults, and in vaccinated children [48,87,107,108].

In addition to their role as immunogens, NSP4 and peptides derived from NSP4 can function as an adjuvant to enhance immune responses for a co-administered antigen. Intranasal co-administration of keyhole limpet hemocyanin, tetanus toxoid, ovalbumin or double layer (VP2/VP6) RLPs with either the full length simian RV SA11 NSP4 or its cleavage product, $NSP4_{aa112-175}$, significantly enhanced antigen-specific systemic and mucosal immune responses in mice [106]. The influenza M2e-antigen fused with the NSP4 coiled-coil region ($NSP4_{aa98-135}$) fragment also showed an increased immunogenicity and protective efficacy of the M2e-antigen. Most importantly, vaccination with M2e-$NSP4_{aa98-135}$ caused a significant decrease in lung virus load early after challenge with influenza A virus and maintained its efficacy against a lethal challenge even at a very low vaccine doses [109].

The structural proteins of the RV outer capsid, VP7 and VP4, are also the main targets for vaccine discovery and development. They independently induce neutralizing antibodies to the virus [66,110,111] and targets for homotypic or heterotypic protective antibodies [111,112]. The glycoprotein, VP7 forms the smooth shell of the virion, while the non-glycosylated VP4 forms the spikes of RV that extend from the viral particle surface (Fig. 2-6A) [113]. Trypsin cleavage of VP4 into VP8 (28 kDa) and VP5 (60 kDa) is required for high-level of viral infectivity and stabilization of the spikes [9]. The VP8 domain binds to cell surface receptors at the early stage of cell-entry and the VP5 facilitates subsequent membrane penetration [3,4]. The trypsin cleavage products remain associated in the virion and consist of a projection with obvious 2-fold symmetry and a trimeric base. The first 26 amino acids of VP8 form α-helix structure, and the N-terminal helices of three subunits (yellow coloured) make up a three-chain coiled-coil which inserts into the foot of the spike contained three VP5 C-terminal domains (green coloured) (Fig. 2-6B and C) [9]. The lectin-like, globular domain of VP8, residues 65-231, forms the distal head of the spike (Fig. 2-6C). The two lectin-like domains at the distal end of the spike are linked to the N-terminal helices by the

segments of residues 27-64 [9,113]. The N-terminal α-helices domain of VP8 contain a linear B-cell epitope [8]. The epitope contains residues 1-10 of VP8, and is highly conserved among different strains of human RVs [8,114]. Three tandem copies of RV10, expressed as a thioredoxin and a universal tetanus toxin T-cell epitope (P2) fusion protein, induced the production of anti-RV10 specific and human RV-neutralizing antibodies in mice [114].

Residues 65-224 of the lectin-like domain of VP8, designated VP8* (Fig. 2-6C), is the distally located domain of the RV spike protein. This domain is essential for initial cell attachment of RV via interactions with specific cellular glycans [9,115,116]. Although many RV strains bind to terminal sialic acid-containing receptors, such as GM3, they can switch between entirely different classes of glycans, such as histo-blood group antigens, via a small changes in the VP8* receptor-binding pocket [117]. Since interference with initial binding of RV with glycans is likely to prevent viral infection, the interactions of cellular-glycans and VP8* can serve as targets for drug discovery or vaccine development. VP8* (18 kDa) is a large and conformational antigenic domain of VP8, and the antibody directed against VP8* blocks binding of the viral particle to the host cell receptors and prevents release of RV DLPs into the cytoplasm of host cells (Fig. 2-6D) [115]. Thus, VP8* has been considered as the best candidate for RV vaccine development.

Several studies have demonstrated the successful induction of RV-neutralizing antibodies using VP8* and VP8-based subunit vaccines [8,114,118,119]. Chickens vaccinated with *E. coli*-expressed VP8* elicited anti-VP8*-specific antibodies which displayed high neutralization activity against the Wa strain of human RV [120]. VP8* antigen expressed in glycosylation deficient *Saccharomyces cerevisiae* induced a strong immune response in female mice that conferred passive protection to their offspring [121]. Wen *et al.* [122] also generated recombinant VP8* protein of human RV strains Wa P[8], DS-1 P[4] and 1076 P[6] in *E. coli* and reported observations of high levels of homotypic and variable levels of heterotypic neutralizing antibodies against each of the antigens when administered to guinea pigs intramuscularly. Their findings indicated that the VP8* proteins may be a plausible additional candidate for new parenteral RV vaccines. In another study, a recombinant VP8* antigen expressed in tobacco transplastomic plants induced protective immunity against bovine rotavirus infection in a suckling mouse model [118]. More recently, the inclusion of a universal tetanus toxoid $CD4^+$ T cell epitope P2 significantly enhanced the immunogenicity of a recombinant RV VP8* subunit parenteral vaccine [123]. In addition, two other truncated VP8 proteins, VP8-1 ($VP8_{aa26-231}$) and VP8-2 ($VP8_{aa51-231}$), were expressed in *E. coli* and evaluated for their immunogenicity and protective efficacy. VP8-1 subunit vaccine showed better yield, homogeneity and high protective efficacy [113]. Favacho *et al.* [111] attempted to produce a high quantity of recombinant bovine RV VP8ext (the extended VP8-including the entire trypsin cleavage

region) in *E. coli* for studying the structure-function relationship of the VP8 fragment in order to understand its role in cell attachment and RV tropism. Although recombinant VP8-based proteins or peptides as subunit vaccines have several advantages over live vaccines, most of them are poorly immunogenic and need toxic adjuvants to boost their immune response. Moreover, their yield from different heterologous expression systems is low for commercial exploitation of the vaccine antigens [96,111,113,114,118,122]. Interestingly, and probably due to the size and conformational sensitivity of VP8*, it has not previously been arrayed on a VLP able to undergo *in vitro* self-assembly.

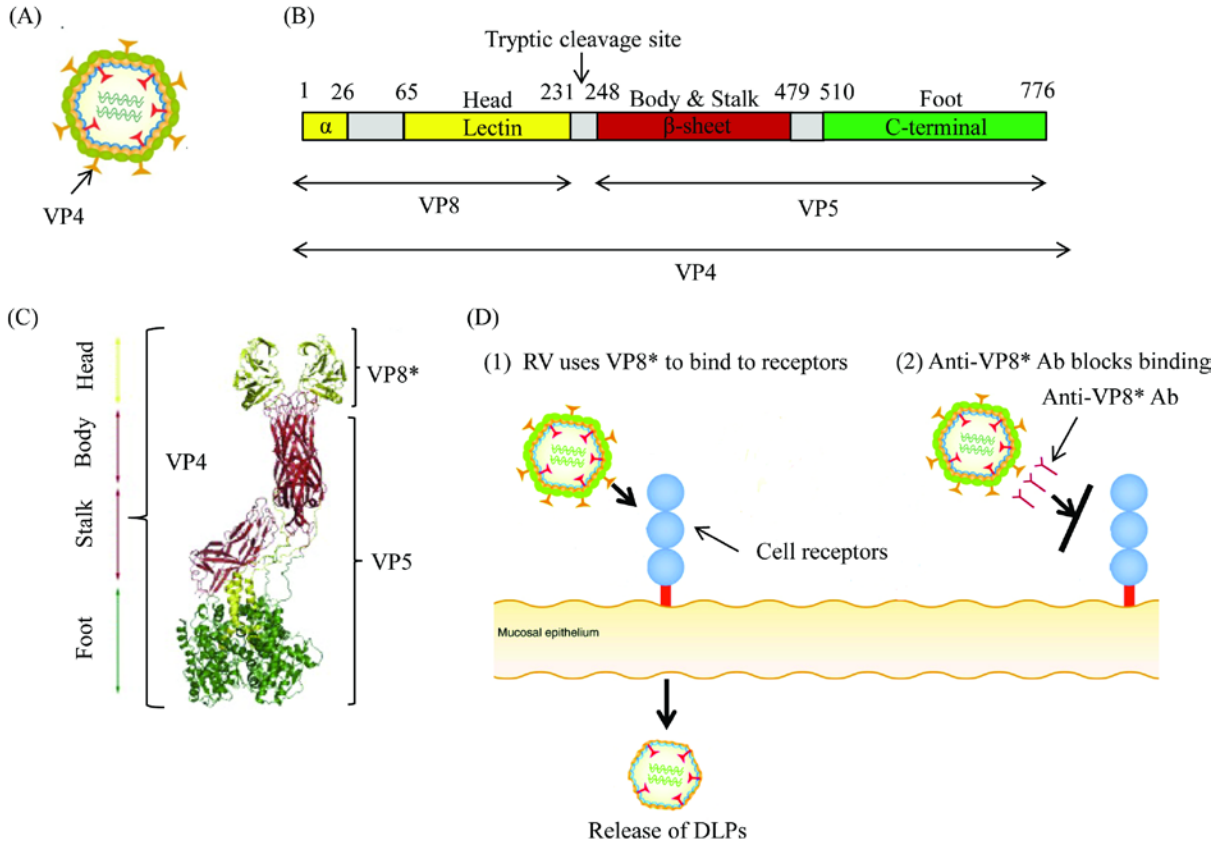

Figure 2-6. Structure of a RV VP4 spike protein and its VP8 subunit domain. (A) Structure of a RV triple layer particle with VP4 spike. (B) The bar representation of domains of VP4. The amino acid residues of different domains of VP4 were labelled above the coloured bar. α : the N-terminal α-helix of VP8 with residues 1-26; residues 27-64 without well resolved structure; lectin: the lectin-like, globular domain of VP8 with residues 65-231; β-sheet: the antigenic domain of VP5 with residues 248-479; the three-chain coiled coil with residues 480-509; and the C-terminal of VP4 with residues 510-776. Tryptic cleavage of VP4 results in VP8 (residues 1-231) and VP5 (residues 248-776) with excision residues 232-247. (C) The ribbon representation of the atomic structure of VP4. The morphology of the spikes in the mature virion described as head, body, stalk and foot. The head contains the lectin-like domain. The β-sheet of the two subunits form the body and that of the third subunit forms the stalk. The foot contains the N-terminus of VP8, the α-domain and the C-terminus of VP5. (D) Interaction of the VP8 subunit domain of VP4 with host cell receptors for viral infectivity. The figure was adapted with slight modifications from Clarke et al. [115](Figure 2-6 A and D) and Xue et al.[113](Figure 2-6 B and C).*

2.6.3.2. Rotavirus-like particles

VLPs are highly organized multimeric protein complexes that self-assemble from viral structural protein(s) and mimic the morphological structure of the corresponding native virus particles without the viral genome, other structural protein(s) and non-structural viral protein(s) [124]. They are produced by the recombinant expression of structural protein(s) in different heterologous expression host cells from bacterial expression systems to various mammalian cell lines [125]. VLPs are undergoing research as tools for vaccination, gene therapy, drug delivery, diagnostics, nanomaterials and for protein interaction and cell interaction studies [124,125]. Particularly, their application in the field of vaccinology has increased interest because of their high safety and efficacy profile, their repetitive and high density native display of epitopes or their self-adjuvanting property, their ability to present foreign epitopes on their surface, their particulate and multivalent nature and their stability compared with soluble antigens [124,126]. They are generally more immunogenic than other subunit vaccines such as recombinant peptide and/or protein immunogens and are able to induce strong humoral and cellular immune responses even without adjuvants [124,126]. Currently, three VLPs, VLP-based hepatitis B virus surface antigen, the human papillomavirus capsid protein L1 and the recombinant VLP-based vaccine Hecolin®, are commercialized for human use against hepatitis B virus infection, human papillomavirus-induced cervical cancer and hepatitis E virus infection respectively [124-126]. Numerous VLP- and chimeric VLP-based vaccines against many infectious agents such as influenza virus, parvovirus, Norwalk, norovirus, Group A streptococcus, RV etc. have shown promising results under clinical trials and pre-clinical evaluations using small animal models [124].

RLPs can be safe and effective alternative candidate vaccines to live-attenuated oral vaccines. It has been also shown that the immunogenic response is higher when the viral proteins are associated into particles than when they exist in linear, soluble form [127]. Studies in different animal models have shown that the different RLPs including double layer (VP2/VP6)-, triple layer (VP2/VP6/VP7)- and complete (VP2/VP4/VP6/VP7)-RLPs showed different better levels of protection after challenge although the protective efficacy depends upon composition of proteins in RLPs, route of administration, the type of the adjuvants and the species of the animals used [127,128]. RLPs are synthetic mimics of the virus. They are produced by simultaneous expression of different recombinant structural proteins in a eukaryotic expression system, such as the insect cell-baculovirus expression vector system [129] and/or yeast expression system [130].

The production of RLPs using the insect cell-baculovirus expression vector system is a highly complex system. The process involves first the expression of the four RV structural proteins, VP2,

VP6, VP7 and VP4, and their subsequent assembly in to RLPs as shown in figure 1-3 (Chapter 1). The process is not completely efficient and unassembled monomers, single-layered and double-layered RLPs are also found in production processes aimed at producing double-layered- or triple-layered-RLPs [131]. This makes the downstream processing and quantification of the desired RLPs so complex and expensive. The yield is very low and scale up also seems infeasible [129,131,132]. Rodriguez-Limas *et al.* [130] have also produced triple-layered RLPs using the yeast, *Saccharomyces cervisiae*, as expression host cell. However, the concentration of the desired RLPs produced inside the yeast cells was very low compared to other expression platforms.

In another study, a RV vaccine candidate using a VLP subunit platform has been reported, based on the combination of truncated VP8* (aa65-aa223) from the RV spike protein with the protrusion (P) domain of the norovirus capsid protein. The resulting chimeric VLP was highly immunogenic although the limitations of yield and scalability can be the challenge of the system as it was made inside a cell, and then purified using low-throughput SEC [133]. Production of VLPs or chimeric VLPs inside the expression host cell is efficient in most cases. However, it is not suitable for a large-scale bioprocessing because of possible contamination of the product with nucleic acids and the formation of heterogeneous VLPs that lead to much more complex downstream processing [134]. Such historical approach to VLP manufacture via *in vivo* assembly of expressed structural proteins inside the expression host cells is very challenging for mass scale production of quality VLPs as vaccines for use in the developing world.

An alternative and now proven approach for VLP assembly via cell-free *in vitro* processing has been developed over a decade for low-cost production of highly purified and quality VLPs [135-139]. Professor Anton Middelberg and Dr Linda Lua, and their groups at The University of Queensland, have devised a scalable and transformational microbial platform technology. Under this approach the murine polyomavirus VP1 and truncated VP1 are produced as soluble recombinant proteins at gram-per-liter levels using *E. coli* cells and followed by purification of capsomeres using chromatographic [138-140] or non-chromatographic methods [141] and/or optional *in vitro* assembly of capsomeres to form VLPs in a cell free reactor. Such platform offers an efficient, homogenous, stable, highly immunogenic product, and significantly reducing scaling-up time and complexity [136-139]. The VP1 capsomere and VLP platforms can serve as a carrier for presentation of immunologically relevant RV antigens for low-cost production of capsomeres and VLPs in a modular format.

2.7. Murine polyomavirus VP1 vaccine platforms

Polyomaviruses are ubiquitous members of the *Polyomavirdae* family that infect both mammalian and avian hosts [142]. They are non-enveloped particles containing a circular, double-stranded DNA genome. The murine polyomavirus is one of the best-characterized species of mammalian polyomaviruses [142,143]. Its genome codes six proteins of which three are structural proteins, which are designated as VP1, VP2 and VP3 [144,145]. The murine polyomavirus VP1 has a molecular mass of 43 kDa and its primary structure contains sequences of 384 amino acids [145]. It is the major capsid protein comprising approximately 80% of the protein found in the capsid of the wild-type virion [146].

Capsids of the murine polyomavirus consist of 360 copies of VP1, that assemble into T= 7 *dextro* (*T, triangulation number*) icosahedral shells. Each capsid shell comprised of 72 pentameric capsid subunits, termed capsomeres, which are arranged in an icosahedral lattice with a diameter of approximately 45 to 50 nm [146,147]. The capsomeres of each capsid are set up in sixty hexavalent and twelve pentavalent capsomeres. Each pentavalent capsomere is encircled by further five capsomeres, whereas each hexavalent capsomeres by further six capsomeres. The carboxy-terminal arms of VP1 stabilize the pentameric capsomeres among each other, whereupon the N-terminus of the VP1 protein contains the viral DNA-binding domain and a nuclear localization sequence [147,148]. The native murine ploymavirus also contains the minor capsid proteins VP2 and VP3 [149]. The VP1 capsomeres are also able to assemble into VLPs *in vitro* in the presence of calcium and specific pH conditions, with a size and morphology similar to the native murine polyomavirus capsids containing the viral genome and minor capsid proteins VP2 and VP3 [150].

DNA-free murine polyomavirus VP1 VLPs have shown promising potentials as vectors for immunotherapy, targeted gene and drug delivery, and as vaccines against viral infections and cancer [134]. As candidate vaccines, VP1-VLPs have shown dramatic effectiveness as they mimic the overall structure of authentic virus particles and present viral antigens in a more authentic conformation than other subunit vaccines [149,151-153]. For a VP1-VLP to be a realistic vaccine candidate and/or delivery vehicle, it needs to be produced in a safe expression system that is amenable to large-scale production [136,151]. For VP1- VLP manufacture, VP1 can be produced using a baculovirus insect cell, a yeast or an *E. coli* expression system [134,136]. Despite the success of the eukaryotic cells in VLP production for a wide variety of viruses, a major limitation with this route occurs because of nucleic acid contamination and the formation of heterogeneous VLPs. In terms of large-scale bioprocessing, these problems may lead to batch-to-batch product variability, making the downstream process much more complex and expensive [134]. To overcome

such limitations, alternative approaches based on microbial transformational platforms have been reported. Using this approach, the murine polyomavirus major capsid protein, VP1 is produced as soluble recombinant glutathione-S-transferase (GST) fusion proteins in *E. coli* cells. Enzyme-mediated release of the GST tag followed by purification and separation by SEC resulted in large amounts of pentameric VP1 capsomeres [136,139]. *In vitro* assembly of highly purified VP1 capsomeres has resulted in formation of quality VLPs through addition of specific agents in a cell-free bioreactor [135,137,139,150].

The N- and C-terminal of VP1 domains are involved in the formation of VLPs [154]. Exclusion of either C-terminal domain or both the N-and C-terminal domains prevented assembly of VP1 capsomeres into VLPs [138,140]. These capsomeres are more stable, and can be used as cheap alternative vaccines to their corresponding VLPs. Both VP1 VLPs and capsomeres can serve as as carrier vaccine platforms for the display of foreign epitopes [138,140,155].

2.7.1. The VLP vaccine platform

Insertion of immunologically relevant vaccine antigens to the viral structural proteins at the DNA level through polymerase chain reaction or gene construction have resulted in modular VLPs [125,156]. Such viral structural proteins, produced at low cost and high yields, able to display the foreign epitopes on their surface, are currently promising alternative vaccine candidates for a number of different diseases [124]. The murine polyomavirus VLPs, which are formed from overexpressed major capsid protein VP1, are among the promising VLP platforms for development of modular VLPs for vaccination [157]. They have high capacity for insertion of peptide epitopes [137,145,157] and have demonstrated huge potential as carrier platforms for the display of foreign epitopes and antigens on the surface in a modular architecture [137,157].

VP1 has two surface exposed loops, S1 loop at amino acid 86 and S4 loop at position 293 (Fig. 2-7) for insertion of foreign antigenic modules [138,145]. Unlike S1 loop, S4 loop has shown high tolerance and capacity for insertion of peptide epitopes [138]. The S4 loop was engineered to contain *Afe*I restriction enzyme sites for molecular insertion of heterologous modules [138]. Insertion of a single module [138] or two tandem modules [155,158] of J8-peptide from the M-protein of Group A streptococcus at S4 loop have resulted in modular VP1 that forms modular VP1 capsomeres when expressed in *E. coli.* Purified modular capsomeres assembled into modular VLPs *in vitro* using a cell-free bioreactor. The modular VLPs showed clear immunogenicity in mice [138,158]. The produced J8-peptide-specific antibodies exerted *in vitro* bactericidal activity [138], and showed protective efficacy in mice [155].

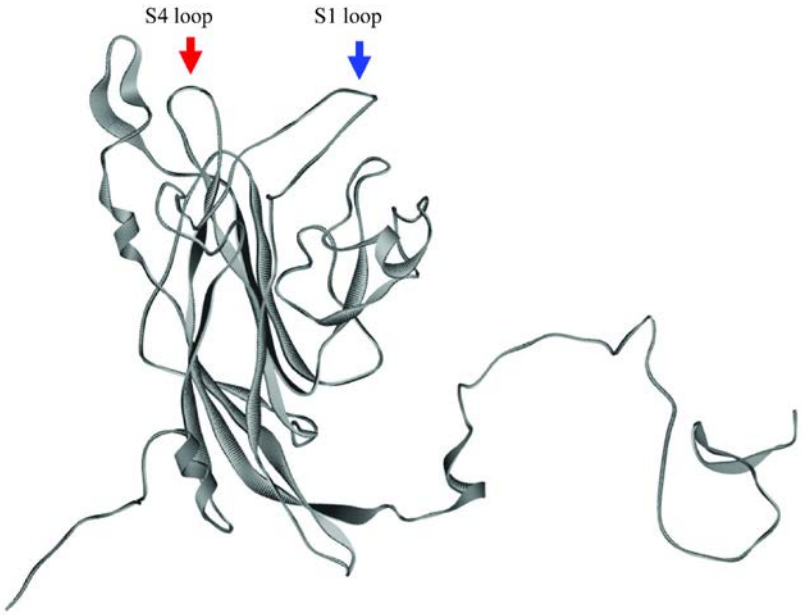

Figure 2-7. The two surface exposed loops of murine polyomavirus VP1.

The VP1 VLP vaccine platform technology summarized in Fig. 2-8 offers an efficient, homogenous, stable, highly immunogenic product, and significantly reducing scaling-up time and complexity [135-139,150]. Its simplicity and ease of scalable will make the VLP vaccine platform an ideal technology in low-and middle-income countries of Africa, Asia and Latin America. It can be used for production of modular vaccine candidates for prevention of various target diseases at low-cost in the developing world.

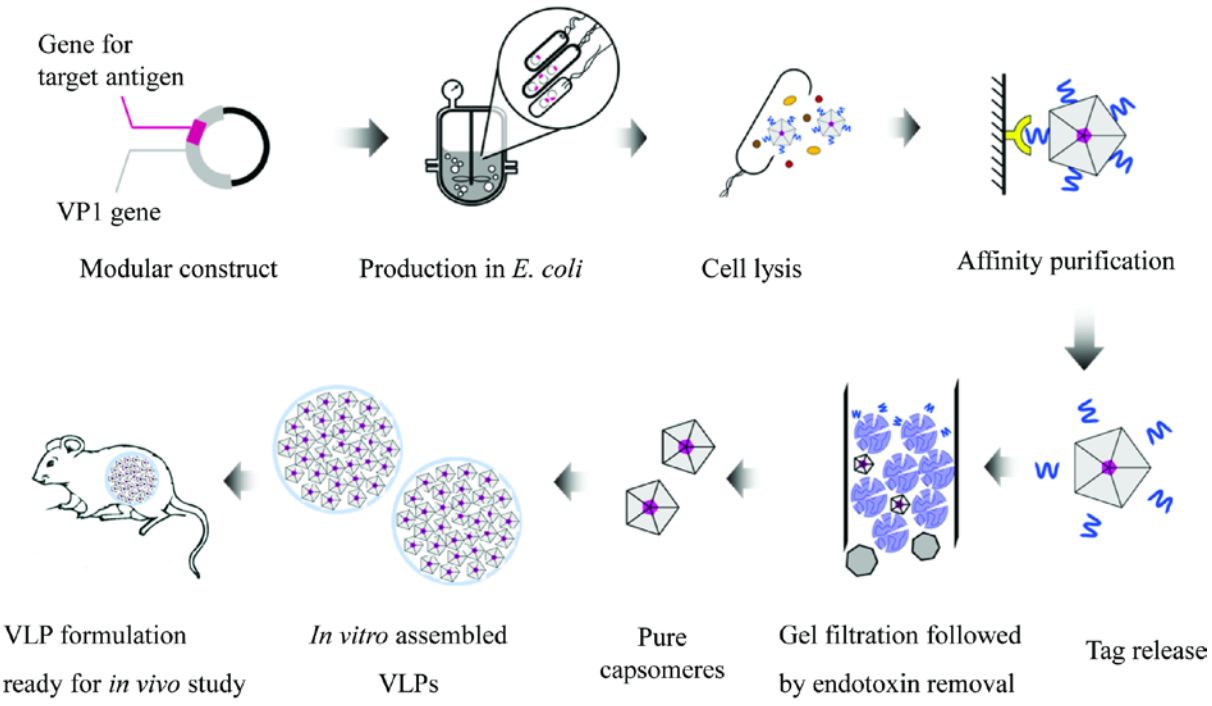

Figure 2-8. Bioprocessing of modular VLPs using the VLP vaccine platform. Insertion of a synthetic gene encoding a target antigen from various pathogens at surface exposed S4 loop of VP1 results a modular construct. The modular construct expresses GST-tagged modular proteins that form pentameric modular capsomeres in E. coli. Cell lysis by sonication or homogenization brings proteins into the extracellular environment. GST-tagged proteins can be captured by affinity chromatography. Enzyme-mediated release of the GST tag followed by purification by gel filtration and endotoxin removal result in highly purified capsomeres. Pure capsomeres can be assembled in vitro into VLPs and VLP formulation can be tested in animals for its immunogenicity and protective efficacy. This figure was produced based on the information available in Middelberg et al. [138].

2.7.2. The capsomere vaccine platform

The N-and C-terminal domains of VP1 are involved in VLP formation [154]. Exclusion of the last 63 amino acid residues from the C-terminal, and the first 28 amino acids from the N-terminal and 63 amino acids from the C- terminal of VP1 result in truncated VP1 (Fig. 2-9C and D). Recombinant expression of truncated VP1 variants in *E. coli* has resulted in the formation of stable pentameric capsomeres that do not assemble *in vitro* into VLPs [138,140]. The capsomeres with engineered insertion sites at their exposed surface loops and C-terminus (Fig. 2-9C) [138] or at their surface exposed loops, N-terminus and C-terminus (Fig. 2-9D) [140] can serve as carrier platforms for heterologous modules in a modular architecture. For example, insertion of a single [138] or multiple [140] modules of M2e- antigen from influenza (A/California/04/ 2009/H1N1) at engineered N (position 28), S4 loop (Position 293) and C (Position 320) of truncated VP1 according to the VP1 amino acid sequence has resulted in expression of modular capsomeres that did not form

VLPs via *in vitro* assembly. Formulation of these modular capsomeres with safe and effective adjuvants showed clear immunogenicity [138,155] and protective efficacy [159] in mice.

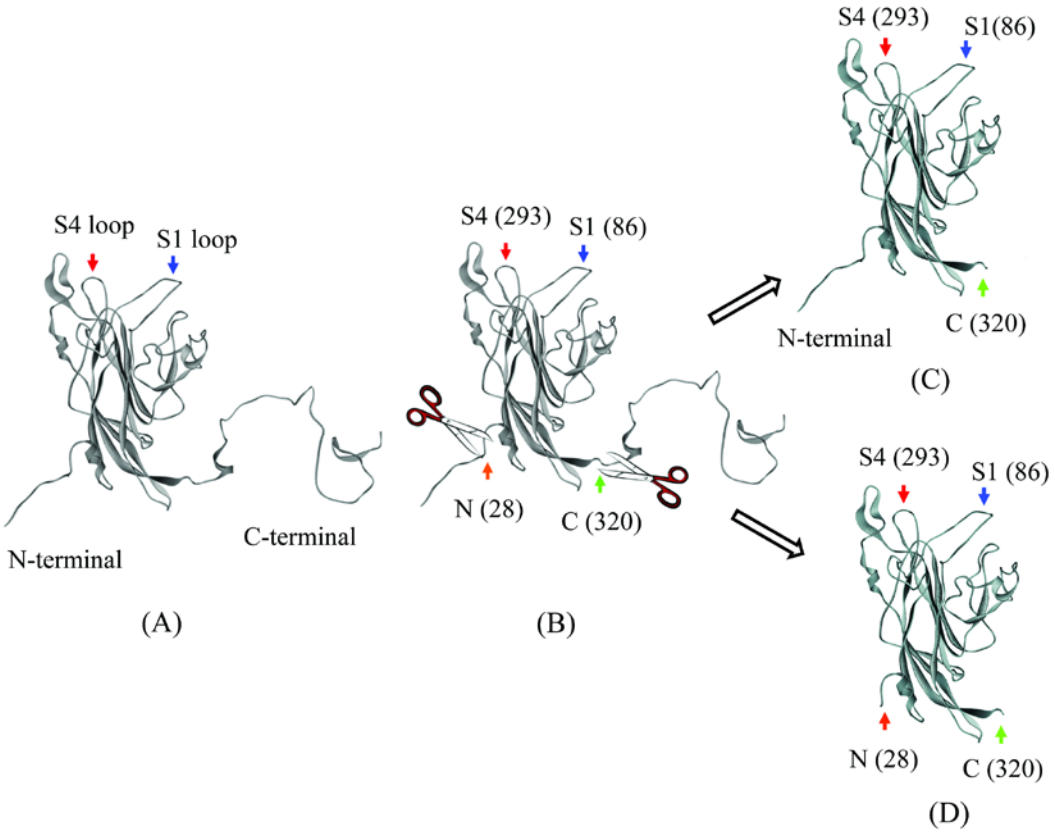

Figure 2-9. Murine polyomavirus VP1 and truncated variants with module insertion sites. (A) The murine polyomavirus VP1 with its surface exposed loops, N-and C-terminal domains. (B) The murine polyomavirus VP1 with its surface exposed loops and sites of truncation for N-and C-terminal domains. (C) Exclusion of the last 63 amino acid residues from the C-terminal resulted in VP1ΔC63. (D) Exclusion of the first 28 amino acid residues from the N-terminal and the last 63 amino acid residues from the C-terminal resulted in VP1ΔN28ΔC63. This figure was produced based on the information available in Middelberg et al. [138]and Wibowo et al. [140].

Modular capsomeres have been produced in high yields from low-cost bacterial expression systems [138,140]. The capsomere platform has received increased interest recently because of its simple, technically less difficult and cheap manufacturing processes (Fig. 2-10) compared to manufacturing of the corresponding modular VLPs (Fig. 2-8). Manufacturability using simple, cheap and efficient processes, and their biological efficacy [159] will make modular capsomeres alternative second-generation cheap vaccines to the corresponding modular VLPs. The manufacturing and processing costs have been decreased further using easily scalable non-chromatographic unit operations, particularly for manufacturing modular capsomere vaccine for veterinary applications [141]. Simplification of technical manufacturing difficulties, improved process efficiency, and

minimization of manufacturing and processing costs often enables low-resource countries to participate in manufacturing processes and/or to use the final product with affordable costs.

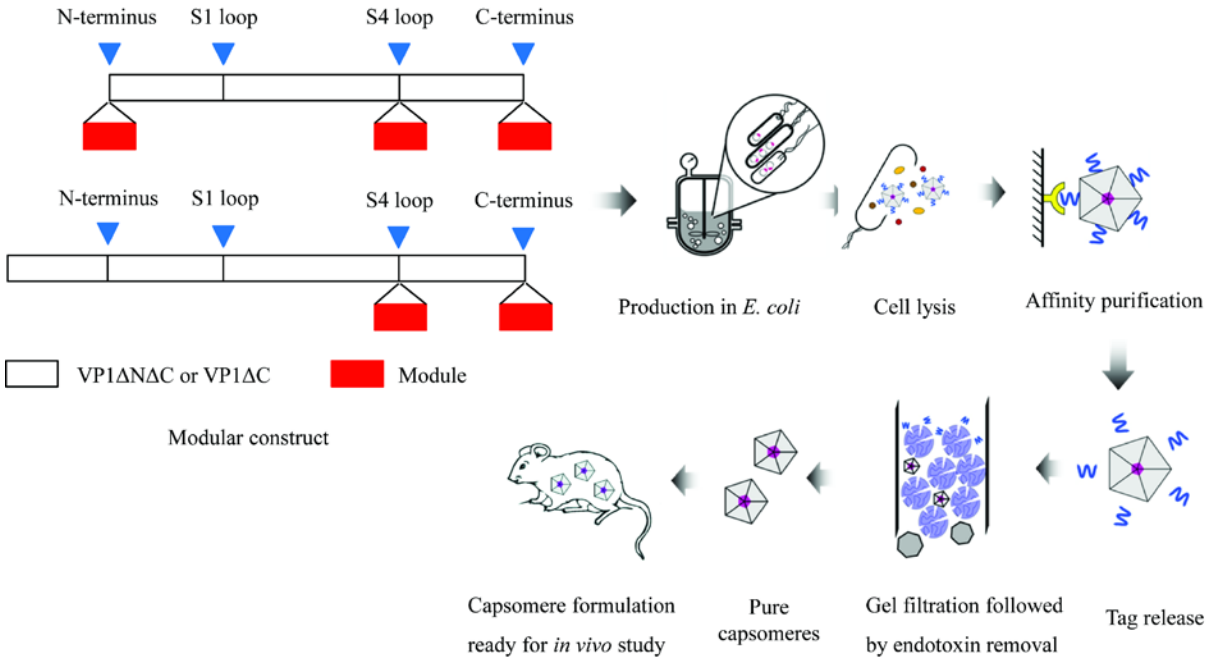

Figure 2-10. Bioprocessing of modular capsomeres using the capsomere vaccine platform. Insertion of a synthetic gene encoding a target antigen from various pathogens at N-terminus, surface exposed S4 loop and/or C-terminus insertion site(s) of truncated VP1 results a modular construct. The modular construct expresses GST-tagged modular proteins that form pentameric modular capsomeres in E. coli. Cell lysis by sonication or homogenization brings proteins into the extracellular environment. GST-tagged proteins can be captured by affinity chromatography. Enzyme-mediated release of the GST tag followed by purification by gel filtration and endotoxin removal result in highly purified capsomeres. Formulation of pure capsomeres can be tested in animals for its immunogenicity and protective efficacy. This figure was produced based on the information available in Wibowo et al. [140].

Modularization of an effective and easily made part of antigenic modules into the capsomere and VLP platforms can be well tolerated [138,140,155]; however success depends on the physicochemical properties, size, density of the inserted modules. Thus, modularization may require optimization of bioprocessing conditions, specific design principles using synthetic biology methods and/or reducing the density of modules to express soluble target modular proteins and to maintain the stability of modular vaccine candidates.

2.8. Strategies for expression of soluble modular proteins and enhanced stability of modular capsomeres and VLPs

Modularization of capsomeres and VLPs may alter protein characteristics such as isoelectric point, hydrophilic-hydrophobic property, secondary structure, three-dimensional structure, the structural

features of protein-protein interfaces and the nature of their interactions, and the interaction of amino acid residues and subdomains of proteins with solvent molecules. Most of these characteristics may depend upon the amino acid composition of the protein, the amino acid sequences and sizes of the inserted modules. It has been widely accepted that the amino acid sequence of a protein determines its three-dimensional structure, dynamics, structural stability and, ultimately, its biological function [160]. Combination of hydrophobic and hydrophilic amino acids is essential for formation and stabilization of protein secondary structure [161]. The capacity and tolerance of VP1 insertion sites for inserting heterologous antigenic modules may also responsible for changes in structural features and physicochemical properties of the proteins. A study demonstrated that large antigens inserted at surface exposed S4 loop of VP1 caused structural perturbations of VLPs [162]. On the other hand insertion of highly hydrophobic antigenic modules on the surface exposed loops may result in high surface hydrophobicity of proteins, which is often associated with changes in protein conformations and related to the ease with which a protein unfolds [163]. Although protein hydrophobicity is very essential for maintaining a protein`s folded structural integrity in cooperative with electrostatic interactions, hydrogen bonds, van der Waals forces and covalent disulphide bonds [164], high surface hydrophobicity due to solvent exposed hydrophobic patches on protein surface may negatively affect expression of soluble proteins in *E. coli* and their stability during expression *in vivo* and/or during downstream processing *in vitro*. Producing soluble proteins for purification and manufacturing of stable proteins is a major concern, particularly for the field of vaccination, where lower efficacy can be a result of poor vaccine stability [165]. A number of approaches for successful expression of soluble proteins and stabilization of the proteins at different stages of processing are described in the literature [166]. Some of these strategies are described below.

2.8.1. Tailoring expression and bioprocessing conditions

Production of recombinant proteins using heterologous expression hosts, such as *E. coli*, is a complex process involving optimization of multiple factors in parallel to obtain soluble, stable and functional proteins [139]. Depending on the physicochemical properties or size specific to each heterologous module, modular constructs may not express target proteins at all or in substantial amount, or express as inclusion bodies, or express soluble protein that form undesired forms, such as protein aggregates, during downstream processing. Insertion of large antigens on a VLP surface caused structural perturbations and resulted in expression of modular proteins that did not form VLPs [162] whereas constructs containing hydrophobic modules often fail to express the target proteins in *E. coli*. Highly hydrophobic proteins may be harmful to recombinant expression host cells and, thus prematurely terminated polypeptides, trapped folding intermediates and partially

folded proteins may be consistently targeted for degradation to avoid their accumulation in cells [167].

Proteolytic degradation of a protein must be prevented to improve the level of its expression. Targeted expression of proteins as inclusion bodies in *E. coli* is one of the methods, gaining considerable success to avoid proteolytic degradation of proteins. It can be achieved using fusion partners specifically designed to enhance over expression of proteins as insoluble inclusion bodies [168]. Particularly, this approach is suitable for the mass scale production of short peptides and inherently disordered proteins. But, other proteins forming inclusion bodies need solubilisation and *in vitro* refolding procedures to obtain a functional protein [169]. Refolding procedures are generally complicated and expensive, and yields are usually low due to re-aggregation of the solubilized polypeptide chains during the refolding process [170]. The *in vitro* refolding may not usually work successfully for all target classes, particularly; recovery of proteins in their pentameric form following refolding procedures may become a challenging and a daunting task for manufacturing of capsomere- and/or VLP-based vaccines. Thus, obtaining soluble proteins, which can maintain their colloidal stability both *in vivo* and *in vitro*, directly from the expression host cells is highly desirable.

The production of soluble proteins in *E. coli* is affected by several factors. Optimization of environmental factors, such as inducer concentration and induction temperature [139,166], induction time [166], growth media components and additives [171-173], can provide dramatic improvements in expression of soluble proteins. Optimization of genetic factors (including *E. coli* strains and expression vectors) [139,174] and other factors, such as lysis buffers and their additives [166], can also tackle protein solubility problems. The attachment of target proteins with fusion tags such as thioredoxin, GST, maltose binding protein, N-utilization substance A and small ubiquitin-like modifier has been commonly used to prevent degradation, promote proper folding and enhance expression of soluble proteins in *E. coli* [175,176]. Some of the fusion partners are also serving as tags for affinity purification to capture soluble fusion proteins [176]. In most cases, release and removal of the tag proteins, particularly, the large fusion tags, are essential to characterize target proteins using size-limited analytical tools [174] and to use the target proteins for *in vivo* application [140,174] or to use the target proteins as a starting material for manufacturing functional materials such VLPs for *in vivo* application [138,140]. Tag release needs protease treatment, which often adds to the cost and complexity of the process [177]. Moreover, some target proteins may form aggregates in solution due to their low solubility following release of the fusion tag [178]. Proteins also form aggregates during processing or upon storage because of many intrinsic or extrinsic factors [179].

Protein aggregation is one of the major causes of physical instability of proteins and it is a challenge common to biological systems, experimental research, medical and industrial applications [179,180]. It is one of the major obstacles in development and commercialization of protein-based biopharmaceuticals for the purpose of prophylactic, therapeutic and diagnostic medicine [180]. The aggregates usually exhibit undesirable characteristics that may lead to reduced or no biological activity, immunotoxicity or other undesired side effects [179]. Thus stabilizations of proteins against aggregation remain one of the most intensive research areas in development of proteins for pharmaceutical, medical and commercial applications. A number of approaches for stabilization of proteins at different stages of processing are described in the literature [181,182]. Among those the simplest and the most common method is tailoring the environment surrounding the proteins [182]. This can be achieved by optimizing solution conditions such as pH, ionic strength [182,183] or by adding stabilizing additives [183]. Several additives [165,184-189] have been studied for their potential to stabilize proteins at different stages of processing, formulation or upon storage.

The stability of proteins against aggregation in different buffer conditions can be monitored using different analytical tools and assay methods such as SEC, light scattering methods, high-throughput ultracentrifugation dispersity sedimentation assay-coupled with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, filtration coupled with SDS-PAGE or western blotting, turbidity assay, asymmetric flow field-flow fractionation (AF4), fluorescence microscopy, circular dichroism, fluorescence and fluorescence lifetime spectroscopy [188,190-194]. In practice, however, there is no ideal and universal analytical tool to monitor accurately and reliably the aggregation status of a given protein of interest. Identification of the most effective additive, pH or ionic strength from a large experimental space for each target protein is laborious and time-consuming using analytical methods with low-throughput capacity [195,196]. Therefore, the use of HTS techniques may provide opportunities for simple and rapid screening for the most effective solution conditions. HTS techniques have become a valuable tool for speeding up process development at various stages of processing biopharmaceuticals [197,198]. Usually, for HTS of the buffer conditions, the analytical methods require short analysis time, low sample consumption, and compatibility with a wide range of buffer components, such as buffering agents, salts and additives [188,193]. For example, DLS measurement, which provides *in situ* analysis within short period of time, has been used for miniaturized HTS of VLP formulation conditions that can be further refined by using AF4 with high resolution but with a low throughput [188].

DLS, also known as photon correlation spectroscopy or quasi-elastic light scattering spectroscopy, is a non-invasive diagnostic tool for particle sizing and determination of particle size distribution of proteins or other polymers in solutions or colloidal dispersions [199-201]. The DLS technique (Fig.

2-11) enables for the direct determinations of the translational diffusion coefficient of scattering units from analysis of time-dependent fluctuations of scattered light intensity arising from particles undergoing random Brownian motion [188,201]. The hydrodynamic radius or the Z-average radius (or cumulant mean) of particles can be calculated from the molecular diffusion coefficient using the Stokes-Einstein equation [201]. Despite its low resolution capacity and high sensitivity to the presence of large particles such as soluble aggregates, DLS provides *in situ* analysis with in short period of time and is suitable for HTS of various solution conditions for enhancing the stability of proteins at different stages during protein manufacturing and processing [188].

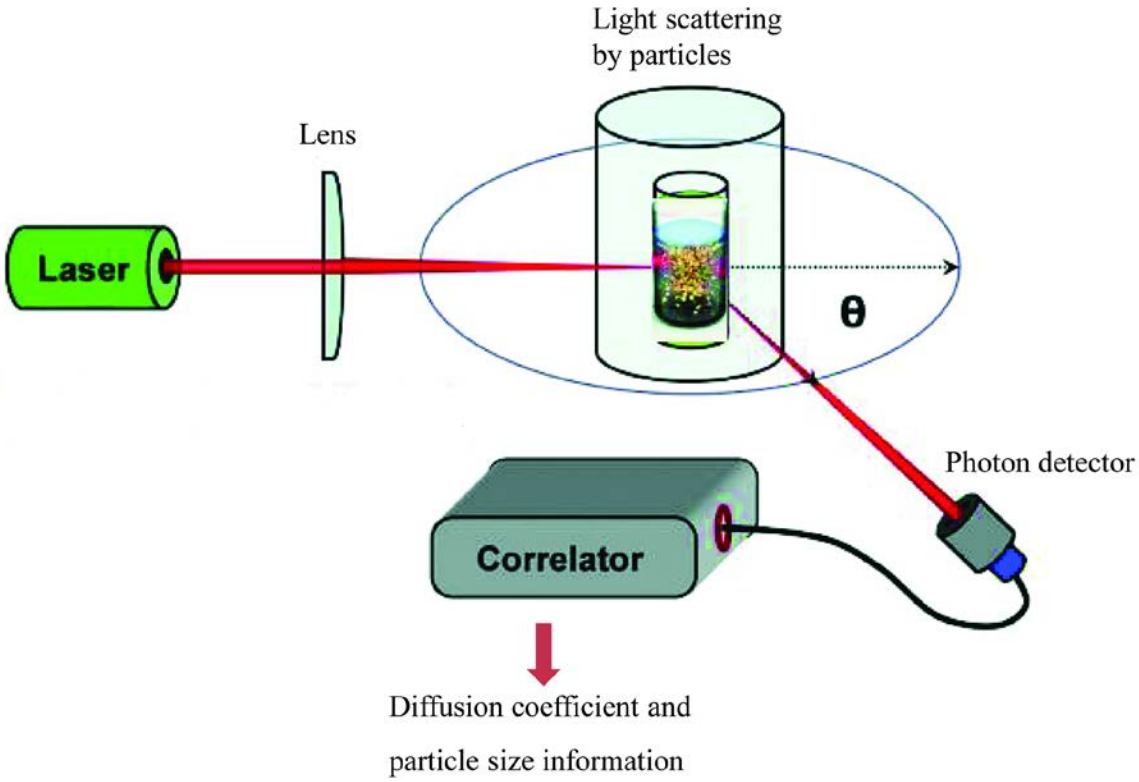

Figure 2-11: The basic working principle of DLS for obtaining diffusion coefficient and particle size information. The sample is illuminated by a laser beam and the fluctuations of the scattered light are detected at a known scattering angle, Θ, by a fast photon detector. Diffusion coefficient and particle size information is obtained from the correlator. This figure was produced with slight modification based on the information available in Li et al. [200].

2.8.2. Synthetic biology design strategies

The advances in DNA synthesis, assembly and sequencing have increased interests in using synthetic biology for the design and/or redesign of individual proteins, protein residues and subunit domains, genes, promoters, operators, terminators, vector replication elements and other core parts of synthetic biology circuits [202]. Using synthetic biology approaches, improved expression

plasmids and, robust and genetically stable expression strains have been developed for production of high yields and titres of target molecules [203,204]. Synthetic biology has the potential to influence various aspects of global health through cheaper drug production, identification and manipulation of host drug targets, manipulation of microbes and their vectors, and developing a range of multivalent vaccines against various target diseases [203]. Synthetic biology tools can enable redesigning of existing biological systems, novel proteins, natural proteins, protein subunit domains and their parts, and synthesizing new ones to deal with specific problems. For example, with the advance of synthetic biology, non-conventional yeasts can be redesigned to expand their role as industrial expression hosts [204]. Conventional expression organisms, such as *E. coli* strains, can be redesigned to facilitate and ease the downstream processing of the target small molecules and proteins [203]. Modular constructs or their modules can be also redesigned to improve protein expression level, expression of soluble protein and to enhance protein stability during expression, purification, formulation and/or storage.

In the design and redesign of modular constructs or their modules for expression of modular murine polyomavirus VP1, the size, physicochemical properties, or number of heterologous modules can be tailored using synthetic biology tools to enhance level of protein expression, protein solubility and stability. Lua *et al*., [162] demonstrated presentation of an 18 kDa RV VP8* large antigen on the surface of VLP using longer linkers. The study revealed that insertion of the longer linkers could ensure structural separation and independence and alleviate structural perturbations of VLPs following insertion of VP8* antigen.

Linkers have played various important roles in recombinant fusion protein production [205]. Flexible linkers are incorporated in fusion protein design to allow interaction between domains or to increase spatial separation between domains whereas rigid linkers are crucial to maintain distance between domains. Cleavable inkers allow *in vivo* separation of protein domains [205]. Linkers showed profound impact on the stability of fusion proteins during expression, processing and storage [206,207]. They are improving protein folding, facilitating protein expression and increasing protein yield [205,208]. They are also modulating the biodistribution and pharmacokinetic profiles of fusion proteins to increase their desired biological activity [205]. G4S is one of the most commonly used flexible linker that is used to maintain inter-domain interactions [155,205]. Longer flexible linkers are able to separate different moieties of fusion proteins spatially in order to alleviate structural perturbation of moieties, which often compromises the stability and activity of fusion proteins [162,206]. Linkers containing polar or charged amino acids often improve solubility of proteins [205].

For modular proteins with high hydrophobic characteristics because of inserted hydrophobic modules, synthetic biology tools enable redesign of the modules to reduce the hydrophobic characteristics of the modules and/or modular proteins that may affect protein expression, solubility and stability. The stability of proteins often depends on hydrophobic interactions, electrostatic interactions, hydrogen bonding and, other intra- and inter-molecular forces between protein molecules and/or protein and solvent molecules [164]. These interactions can be systematically balanced and kept optimum by introducing additional hydrogen bonds on the surface, reducing surface hydrophobicity or through optimization of surface charge or by adding polar or charged amino acids in the sequence of surface exposed residues. A study showed that glutamic acid, aspartic acid and serine contribute most favourably to protein solubility at high net charge [209]. Insertion of double aspartic acid residues into a hydrophobic module enhanced expression of soluble modular murine polyomavirus VP1 proteins [210]. Incorporation of glutamic acid residues (E4) in module design as ionic flanking elements has improved expression of soluble modular VP1 protein inserted with a hydrophobic module from influenza A virus [211]. Stubenrauch *et al.* [212] inserted an octa-glutamic acid peptide as a purification tag in a surface exposed loop of pentameric VP1 of polyomavirus. The polyionic tag was efficiently used to capture pentameric VP1 by ion exchange chromatography and did not inhibit *in vitro* assembly of VP1 pentamers into VLPs. Incorporation of poly ionic peptides into hydrophobic modules may counteract the effect of high surface hydrophobicity on protein solubility, as well as stability against aggregation.

2.8.3. Protein co-expression

Numerous proteins exist in the form of a protein complex containing only two or three to multiple-subunits [213,214]. Most of their subunits exert their functions through formation of active and stable protein complexes, and protein-protein interactions [213]. Traditionally, multi-protein complexes are produced by overexpression and purification of each subunit protein separately using the bacterial, yeast, insect or mammalian host expression system, followed by *in vitro* reconstitution of partner subunit proteins [215,216]. This approach has been successfully used to produce many protein complexes [215]. However, *in vitro* reconstitution often requires refolding procedures at least for one-subunit of the complex and it also suffers from protein aggregation that affects the yield and functionality of the protein complexes or their subunits [215,216]. Protein co-expression technology using either a prokaryotic or eukaryotic expression hosts has become an important strategy for producing a variety of protein complexes for biochemical, biophysical, functional, structural, and high throughput screening studies [215,217]. Unlike the *in vitro* reconstitution or refolding of the individually expressed partners, co-expression usually overcomes solubility

problems of individually expressed subunits and allows their proper folding into their native form, and leads formation of a soluble complex *in vivo* [216,218].

Many protein co-expression systems are available for simultaneous expression of two or more proteins in eukaryotic and prokaryotic cells [219,220]. Protein co-expression in eukaryotic cells may be favoured in case of proteins or their complexes that require post translational modifications for their function and/or stability [216]. Despite the advantages of the eukaryotic systems, co-expression strategies in *E. coli* remain one of the most powerful and the primary systems of choice for production of protein complexes [216,220]. *E. coli* co-expression system has the benefit of obtaining large quantities of target proteins at low-cost and short time due to ease of its manipulation and rapid growth [216,219,220]. Moreover, some successful efforts in enhancing the ability of *E. coli* to secrete proteins to the extracellular environment, efforts in developing antibiotics-free plasmid systems with other alternative methods for selections, and engineering glycosylation and other post-translational modification in to the *E. coli* expression/co-expression systems have born fruits in practical sense for low-cost manufacturing of proteins [221]. For example, Sommer *et al.* [222] demonstrated efficient production of extracellular proteins, such as alkaline phosphatase and β-lactamase, by means of optimized co-expression of bacteriocin release proteins in *E. coli*. A study also showed overexpression of post-translationally modified peptides in *E. coli* by co-expression with modifying enzymes [223].

Protein co-expression has also got potential applications for productions of toxic, membrane or hydrophobic proteins, which often requires the presence of their binding-partner protein(s) to ensure their expression, and their correct folding and stability [213]. Co-expression membrane proteins with folding modulators and chaperones have resulted in production of membrane proteins in large quantities *in vivo* [224]. Studies demonstrated that co-expression of appropriate folding accessory proteins have significantly improved the productivity of active form of proteins in *E. coli* [225,226]. Co-expression of proteins with molecular chaperones have reduced the aggregation of proteins, increased their expression as soluble proteins and activity [227], and improved conformational quality of recombinant proteins in *E. coli* [228]. Protein co-expression has offered an important strategy for improving the yield, activity and stability of different enzymes for structural studies [229], for industrial [218,230,231] and therapeutic applications [217]. Co-expression of structural proteins of complex viruses, such as rotavirus, in eukaryotic cells [129,130] has resulted in the formation of VLPs.

Protein co-expression in *E. coli* and eukaryotic cells can be achieved using either multiple or single expression vector strategies [216,219]. Multiple vector strategy utilizes two or more plasmids, each

carrying the gene(s) expressing one or two component proteins [213,215]. This strategy often requires less cloning steps and provides flexibility when many combinations of individual constructs need to be tested [217]. The expression constructs for each component can be generated in parallel, and used in expression trial and expression optimization individually or in combination [215]. For successful co-expression of proteins in *E .coli* via multiple vector strategy, ideally each vector should contain a different antibiotic selection marker as well as a different origin of replication, in order to stably maintain the plasmids in the host cells [215-217]. However, plasmids with the same origin of replication but with different antibiotic selection markers could co-exist in the cells by simply using higher concentrations of antibiotics [215,217]. For example, commercially available Novagen Duet vectors are used for co-expression of proteins using multiple vector strategy. These vector systems utilize more than two plasmids, each with a different combination of a compatible replicon and an antibiotic selection marker, or simply utilize plasmids with identical, incompatible replicons and different antibiotic selection markers [232]. On the other hand, protein co-expression from a single vector system in *E. coli* can be achieved via either a single expression cassette or multiple expression cassettes, where an expression cassette consists of a single transcriptional unit with "promoter-open reading frame(s) (ORF(s))-terminator sequence elements [215]. With the single-cassette approach, an unrestricted number of genes are inserted into a plasmid to form a single transcriptional unit with either two translational units (bicistronic) or multiple translational units (polycistronic) [213,215,233]. These operon-like constructs have been successfully used for co-expression of two or more proteins under the control of a single promoter [217,232]. The single-cassette approach is the method of choice in cases where strict control of stoichiometry is essential to form a functional protein subunit [213]. Usually, the upstream ORFs are expressed more efficiently than the downstream ones, resulting in sub-stoichiometric arrangement of the resultant complex [215,233]. The second approach for co-expression of proteins using a single vector system relies on tandem coding regions involving multiple expression cassettes [215,217]. Using this approach, individual genes/ORFs are expressed separately under the control of multiple expression cassettes carried by a single plasmid, where in general the number of cassettes is the same as the number of genes transcribed [215,217,233]. This co-expression approach might offer better yield of protein-protein complexes compared to co-expression using multiple vector system and single vector system with a single expression cassette [217,233]. However, it also might lead to an imbalanced ratio of expressed protein products because of uncoupled transcription of the genes, differences in the rate of transcription, translation, translocation, and the stability of RNA and protein products [215,217].

A variety of single co-expression vectors are available commercially for co-expression of proteins using both *E. coli* and eukaryotic cells [215]. Construction of single co-expression constructs usually requires laborious and time-consuming cloning steps to avoid unintended restriction digestion during sub-cloning [215,232]. It is also difficult to change the combination of targeted proteins after generation of a certain plasmid for single vector co-expression strategy [232]. Despite the sequential steps needed for single vector construction, expression of all component proteins from a single co-expression construct often facilitates easy establishment of expression strains and ensures expression of component proteins in the same host cell [215,217,232]. It can also be used for titrating down the ratio of one or more than one components in a protein complex to achieve the desired physicochemical properties of the protein complex that is essential for its physical, chemical and structural stability and biological activity. For example, Lua *et al*. [162] developed a baculovirus-insect cell bicistronic expression system that co-expresses unmodified murine polyomavirus VP1 and modular VP1 presenting an 18 kDa RV VP8* large antigen from a single plasmid. This approach was able to overcome steric hindrance to VLP formation by titrating down the ratio of modular VP1 during co-expression and reducing the amount of the large VP8* antigen on the VLP surface. Due to the shortcomings of manufacturing of VLPs from eukaryotic expression systems, VLP manufacturing via *in vitro* assembly of VLP subunits in a cell-free bioreactor has got huge potential for reduction of manufacturing and processing costs [234]. Chapter 5 addresses extrapolation of the baculovirus-insect cell bicistronic expression system to the bacterial expression system for low-cost production of modular capsomeres, which can be used as alternative vaccine candidates to VLPs or as starting subunits for *in vitro* VLP assembly using a cell-free bioreactor.

2.9. Assessing the immunogenicity and efficacy of RV subunit vaccine candidates *in vivo*

Development of an effective vaccine against RV hinges on an improved understanding of the host immune response to RV. An ideal subunit vaccine candidate should be able to stimulate the appropriate arm of the immune system with concomitant generation of the memory cells. Despite tremendous progress in understanding the structural features [39], the replication cycle [20], mechanisms of rotavirus pathogenesis [33] and immunity [39], full correlation of immune response with protective immunity against RV infection and diarrhoea is not well understood. Understanding the nature of protective immunity against RV will increase the success of developing effective vaccines.

New vaccine development involves a multistep process. The pre-clinical studies are the earliest phases of new vaccine development that entail investigation of candidate vaccines both *in vitro* in laboratories and *in vivo* in animals [235]. *In vitro* studies comprise assessment of quality control of

the manufacturing process and validation of *in vitro* immunogenicity assays to be used in subsequent preclinical and clinical trials. *In vitro* studies are also fundamental components of vaccine design. For example, neutralization assays are employed for identification of the serotype of the most prevalent circulating viral strains, which in turn affects vaccine design and vaccine formulation [235]. As part of the *in vitro* studies in developing non-replicating vaccine candidates against RV, various subunit vaccines containing RV VP4, VP7, VP6 or NSP4 peptides and proteins [5,6,88,113,114], RLPs [129] or modular VLPs containing RV antigens [133,162] have been produced using prokaryotic and/or eukaryotic expression systems.

In vivo studies are designed during pre-clinical evaluation to assess safety and immunogenicity of RV subunit vaccine candidates. Such studies have relied on the use of appropriate animal models to generate valuable safety and immunogenicity data to select high ranking and potential vaccine candidates for clinical evaluation in humans. The role of various animal models for preliminary evaluation of the immunogenicity of new RV subunit vaccine candidates is again an unanswered question, as high immunogenicity in any of these animals provides no assurance of protection either in animals or in humans. Among others, mice, rat, rabbit, piglet, calf, lamb, non-human primates have been used for assessing the safety, immunogenicity and efficacy of RV vaccines [44,236,237]. Mice are extensively used for studying the immunogenicity of different RV subunit vaccine candidates [237]. They are preferred to other animal species because of simplicity of keeping, breeding and reproducing them [238], their small size, low cost, availability of a large collection of inbred, outbred, immunologically deficient and genetically altered RV naïve mice strains, as well as broad accessibility of research immunological reagents [239]. In pre-clinical studies, the outcomes from preliminary immunogenicity assessments are typically viewed as tests of biological concepts for selecting a potential candidate for further clinical studies. Immunogenicity endpoints must be determined in consideration of various factors, such as host and vaccine factors, the type of the desired immune responses-humoral, cellular, or mucosal- and the time points when the immune responses are measured [235,240]. Antigen-specific antibody response usually constitutes the primary immunogenicity endpoint during preclinical study. A qualitative assay such as the dot blotting analysis [241] or a quantitative assay such as the enzyme-linked immunosorbent assay is employed to detect or to measure antibody response [235,242]. Depending on the type of epitopes present in each vaccine candidate, consideration of the cell-mediated immune responses during the early pre-clinical evaluations is also equally important for selecting potential vaccine candidates that can induce protective immunity against RV.

Vaccine candidates with valuable safety and immunogenicity data based on the outcomes from the pre-clinical studies can be used for further efficacy study using different animal models. The adult

mice and rabbit RV infectious models, the gnotobiotic piglet RV disease model, and neonatal mice and calves passive protection models have been used to evaluate protective efficacy of RV subunit vaccine candidates [243]. Studies using the neonatal mice and calf models demonstrated passive protective immunity in mouse pups and calves [244,245]. Efficacy studies of various RV subunit vaccines in adult mice [95,246] and rabbit [44,100,247] models also showed complete or significant partial protection against RV infection, but not against diarrhoea. In comparison, the neonatal gnotobiotic piglet's model presents a number of advantages for evaluating vaccine efficacy. For example, the gnotobiotic pigs closely resemble humans in gastrointestinal physiology and in the development of mucosal immunity, and susceptible up to at least 8 weeks of age to infection and diarrhoea with many human RV strains [236]. Different RV subunit vaccines showed protective efficacy during preclinical studies using the neonatal gnotobiotic piglet's infection and disease model [44,51,128]. Generally, evaluation of the immunogenicity, safety and efficacy of vaccine candidates during preclinical studies helps selection of potential vaccine candidates for further phase I, phase II and phase III clinical studies. Particularly, the efficacy data obtained using neonatal gnotobiotic piglet's model offers information that may be more relevant for the development of RV vaccines for humans. However, the use of the neonatal gnotobiotic piglet's model has been limited in early preclinical studies because of high cost and the need for specialized equipment, facilities and staff [243]. Thus, the mouse model remains as a simple and cost effective choice in the earliest phase of vaccine development. *In vivo* study using the mouse model is particularly suited for preliminary assessment of the immunogenicity of new vaccine candidates. More recently, the mouse model has been used successfully to obtain relevant immunogenicity and protective efficacy data for murine polyomavirus VP1 modular capsomeres and VLPs presenting M2e- and J8-peptide modules, respectively [138,140,155,159]. Despite the presence of a strong pre-existing anti-VP1 antibody, high levels J8i-specific antibody titer was obtained from mice immunized by modular VLPs presenting J8i-peptide modules [158]. These results and the findings in Chapter 5 and Appendix B strengthen the use of the mouse model in the earliest phases of new vaccine development.

References

1. Greenberg HB, Estes MK: **Rotaviruses: from pathogenesis to vaccination**. *Gastroenterology* 2009, **136**:1939-1951.
2. Estes M, Cohen J: **Rotavirus gene structure and function**. *Microbiological Reviews* 1998, **53**:410-449.
3. Arias CF, Isa P, Guerrero CA, Méndez E, Zárate S, López T, Espinosa R, Romero P, López S: **Molecular biology of rotavirus cell entry**. *Archives of Medical Research* 2002, **33**:356-361.
4. Jayaram H, Estes MK, Prasad BVV: **Emerging themes in rotavirus cell entry, genome organization, transcription and replication**. *Virus Research* 2004, **101**:67-81.
5. Ward RL, McNeal MM: **VP6: a candidate rotavirus vaccine**. *Journal of Infectious Diseases* 2010, **202**:S101-S107.
6. Xie L, Yan M, Wang X, Ye J, Mi K, Yan S, Niu X, Lia H, Sun M: **Immunogenicity and efficacy in mice of an adenovirus-based bicistronic rotavirus vaccine expressing NSP4 and VP7**. *Virus Research* 2015, **210**:298-307.
7. Song XF, Hao Y: **Adaptive evolution of rotavirus VP7 and NSP4 genes in different species**. *Computational Biology and Chemistry* 2009, **33**:344-349.
8. Kovacs-Nolan J, Yoo DW, Mine Y: **Fine mapping of sequential neutralization epitopes on the subunit protein VP8 of human rotavirus**. *Biochemical Journal* 2003, **376**:269-275.
9. Settembre EC, Chen JZ, Dormitzer PR, Grigorieff N, Harrison SC: **Atomic model of an infectious rotavirus particle**. *European Molecular Biology Organization Journal* 2011, **30**:408-416.
10. Matthijnssens J, Otto PH, Ciarlet M, Desselberger U, Ranst MV, Johne R: **VP6-sequence-based cut-off values as a criterion for rotavirus species demarcation**. *Archives of Virology* 2012, **157**:1177-1182.
11. Kirkwood C: **Genetic and antigenic diversity of human rotaviruses: potential impact on vaccination programs**. *The Journal of Infectious Diseases* 2010, **202**:S43-S48.
12. Matthijnssens J, Ciarlet M, Rahman M, Attoui H, Bányai K, Estes MK, Gentsch JR, Iturriza-Gómara M, Kirkwood C, Martella V, et al.: **Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments**. *Archives of Virology* 2008, **153**:1621-1629.
13. Matthijnssens J, Ciarlet M, McDonald S, Attoui H, Banyai K, Brister J, Buesa J, Esona M, Estes M, Gentsch J, et al.: **Uniformity of rotavirus strain nomenclature proposed by the**

Rotavirus Classification Working Group (RCWG). *Archives of Virology* 2011, **156**:1397-1413.

14. Matthijnssens J, Bilcke J, Ciarlet M, Martella V, Banyai K, Rahman M, Zeller M, Beutels P, Van Damme P, Van Ranst M: **Rotavirus disease and vaccination: impact on genotype diversity**. *Future Microbiology* 2009, **4**:1303-1316.
15. Bányai K, László B, Duque J, Steele A, Nelson E, Gentsch J, Parashar U: **Systematic review of regional and temporal trends in global rotavirus strain diversity in the pre rotavirus vaccine era: insights for understanding the impact of rotavirus vaccination programs**. *Vaccine* 2012, **30**:A122-A130.
16. Sanchez-Padilla E, Grais RF, Guerin PJ, Steele AD, Burny ME, Luquero FJ: **Burden of disease and circulating serotypes of rotavirus infection in sub-Saharan Africa: systematic review and meta-analysis**. *Lancet Infectious Diseases* 2009, **9**:567-576.
17. Doro R, Laszlo B, Martella V, Leshem E, Gentsch J, Parashar U, Banyai K: **Review of global rotavirus strain prevalence data from six years post vaccine licensure: is there evidence of strain selection from vaccine pressure?** *Infection Genetics and Evolution* 2014, **28**:446-461.
18. McDonald SM, Patton JT: **Assortment and packaging of the segmented rotavirus genome**. *Trends in Microbiology* 2011, **19**:136-144.
19. Wang H, Moon S, Wang Y, Jiang B: **Multiple virus infection alters rotavirus replication and expression of cytokines and toll-like receptors in intestinal epithelial cells**. *Virus Research* 2012, **167**:48-55.
20. Trask SD, McDonald SM, Patton JT: **Structural insights into the coupling of virion assembly and rotavirus replication**. *Nature Reviews Microbiology* 2012, **10**:165-177.
21. Eichwald C, Arnoldi F, Laimbacher AS, Schraner EM, Fraefel C, Wild P, Burrone OR, Ackermann M: **Rotavirus viroplasm fusion and perinuclear localization are dynamic processes requiring stabilized microtubules**. *PloS One* 2012, **7**:e47947.
22. Patton JT, Carpio RV-D, Tortorici MA, Taraporewala ZF: **Coupling of rotavirus genome replication and capsid assembly**. *Advances in Virus Research* 2007, **69**:167-201.
23. Taraporewala ZF, Patton JT: **Nonstructural proteins involved in genome packaging and replication of rotaviruses and other members of the *Reoviridae***. *Virus Research* 2004, **101**:57-66.
24. Donker NC, Kirkwood CD: **Selection and evolutionary analysis in the nonstructural protein NSP2 of rotavirus A**. *Infection Genetics and Evolution* 2012, **12**:1355-1361.
25. Jayaram H, Taraporewala Z, Patton JT, Prasad BVV: **Rotavirus protein involved in genome replication and packaging exhibits a HIT-like fold**. *Nature* 2002, **417**:311-315.

26. Grimwood K, Lambert SB, Milne RJ: **Rotavirus infections and vaccines: burden of illness and potential impact of vaccination**. *Paediatric Drugs* 2010, **12**:235-256.

27. Khoury H, Ogilvie I, El Khoury AC, Duan Y, Goetghebeur MM: **Burden of rotavirus gastroenteritis in the Middle Eastern and North African pediatric population**. *BMC Infectious Diseases* 2011, **11**:DOI: 10.1186.

28. Kim HJ, Park JG, Alfajaro MM, Kim DS, Hosmillo M, Son KY, Lee JH, Bae YC, Park SI, Kang MI, et al.: **Pathogenicity characterization of a bovine triple reassortant rotavirus in calves and piglets**. *Veterinary Microbiology* 2012, **159**:11-22.

29. Okadera K, Abe M, Ito N, Morikawa S, Yamasaki A, Masatani T, Nakagawa K, Yamaoka S, Sugiyama M: **Evidence of natural transmision of group A rotavirus between domestic pigs and wild boars (Sus scrofa) in Japan**. *Infection Genetics and Evolution* 2013, **20**:54-60.

30. Lundgren O, Svensson L: **Pathogenesis of rotavirus diarrhea**. *Microbes and Infection* 2001, **3**:1145-1156.

31. Ball JM, Mitchell DM, Gibbons TF, Parr RD: **Rotavirus NSP4: a multifunctional viral enterotoxin**. *Viral Immunology* 2005, **18**:27-40.

32. Ball JM, Tian P, Zeng CQY, Morris AP, Estes MK: **Age-dependent diarrhea induced by a rotaviral non-structural glycoprotein**. *Science* 1996, **272**:101-104.

33. Bomsel M, Alfsen A: **Entry of viruses through the epithelial barrier: pathogenic trickery**. *Nature Reviews Molecular Cell Biology* 2003, **4**:57-68.

34. Hyser JM, Estes MK: **Rotavirus vaccines and pathogenesis: 2008**. *Current Opinion in Gastroenterology* 2009, **25**:36-43.

35. Lorrot M, Vasseur M: **How do the rotavirus NSP4 and bacterial enterotoxins lead differently to diarrhea?** *Virology Journal* 2007, **4**:1-6.

36. Tate JE, Burton AH, Boschi-Pinto C, Steele AD, Duque J, Parashar UD: **2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis**. *Lancet Infectious Diseases* 2012, **12**:136-141.

37. Liu L, Oza S, Hogan D, Perin J, Rudan I, Lawn JE, Cousens S, Mathers C, Black RE: **Global, regional, and national causes of child mortality in 2000–2013, with projections to inform post-2015 priorities: an updated systematic analysis**. *The Lancet* 2015, **385**:430-440.

38. Angel J, Franco MA, Greenberg HB: **Rotavirus immune responses and correlates to protection**. *Current Opinion in Virology* 2012, **2**:419-425.

39. Angel J, Franco MA, Greenberg HB: **Rotavirus vaccines: recent developments and future considerations**. *Nature Reviews Microbiology* 2007, **5**:529-539.

40. Arnold MM, Patton JT: **Rotavirus antagonism of the innate immune response**. *Viruses* 2009, **1**:1036-1056.

41. Barro M, Patton JT: **Rotavirus NSP1 subverts innate immune response by inducing degradation of IFN regulatory factor 3**. *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**:4114-4119.

42. Qin L, Ren L, Zhuo Z, Lei X, Chen L, Xue Q, Liu X, Wang J, Hung T: **Rotavirus NSP1 antagonizes innate immune response by interacting with retinoic acid inducible gene I**. *Virology Journal* 2011, **8**:1-9.

43. Sherry B: **Rotavirus and reovirus modulation of the interferon response**. *Journal of Interferon and Cytokine Reasearch* 2009, **29**:559-567.

44. Desselberger U, Huppertz HI: **Immune responses to rotavirus infection and vaccination and associated correlates of protection**. *Journal of Infectious Diseases* 2011, **203**:188-195.

45. Ishida SI, Feng N, Gilbert JM, Tang B, Greenberg HB: **Immune responses to individual rotavirus proteins following heterologous and homologous rotaviru infection in mice**. *The Journal of Infectious Diseases* 1997, **175**:1317-1323.

46. Franco MA, Angel J, Greenberg HB: **Immunity and correlates of protection for rotavirus vaccines**. *Vaccine* 2006, **24**:2718-2731.

47. Estes MK, Desselberger U: **Rotaviruses: cause of vaccine-preventable disease yet many fundamental questions remain to be explored**. *Current Opinion in Virology* 2012, **2**:369-372.

48. Yuan L, Ishida S-I, Honma S, Patton JT, Hodgins DC, Kapikian AZ, Hoshino Y: **Homotypic and hetrotypic serum isotype-specific antibody responses to rotavirus nonstructural protein 4 and viral protein VP4, VP6 and VP7 in infants who recieved selected live oral rotavirus vaccines**. *The Journal of Infectious Diseases* 2004, **189**:1834-1845.

49. Parez N: **Rotavirus gastroenteritis: why to back up the development of new vaccines?** *Comparative Immunology, Microbiology and Infectious Diseases* 2008, **31**:253-269.

50. Hou Z, Huang T, Huan Y, Pang W, Meng M, Wang P, Yang M, Jiang L, Cao X, Wu KK: **Anti-NSP4 antibody can block rotavirus-induced diarrhea in mice**. *Journal of Pediatric Gastroenterology and Nutrition* 2008, **46**:376-385.

51. Yuan L, Wen K, Azevedo MSP, Gonzalez AM, Zhang W, Saif LJ: **Virus-specific intestinal IFN-gamma producing T cell responses induced by human rotavirus infection and vaccines are correlated with protection against rotavirus diarrhea in gnotobiotic pigs**. *Vaccine* 2008, **26**:3322-3331.

52. Kaufhold RM, Field JA, Caulfield MJ, Wang S, Joseph H, Wooters MA, Green T, Clark HF, Krah D, Smith JG: **Memory T-cell response to rotavirus detected with a gamma interferon enzyme-linked immunospot assay**. *Journal of Virology* 2005, **79**:5684-5694.

53. Kim B, Feng N, Narvaez CF, He X-S, Eo SK, Lim CW, Greenberg HB: **The influence of CD4(+) CD25(+) Foxp3(+) regulatory T cells on the immune response to rotavirus infection**. *Vaccine* 2008, **26**:5601-5611.

54. Azevedo MSP, Yuan L, Pouly S, Gonzales AM, Jeong KI, Nguyen TV, Saif LJ: **Cytokine responses in gnotobiotic pigs after infection with virulent or attenuated human rotavirus**. *Journal of Virology* 2006, **80**:372-382.

55. Malik J, Gupta SK, Bhatnagar S, Bhan MK, Ray P: **Evaluation of IFN-gamma response to rotavirus and non-structural protein NSP4 of rotavirus in children following severe rotavirus diarrhea**. *Journal of Clinical Virology* 2008, **43**:202-206.

56. Basnet S, Mathisen M, Strand TA: **Oral zinc and common childhood infections-an update**. *Journal of Trace Elements in Medicine and Biology* 2015, **31**:163-166.

57. Munos MK, Walker CLF, Black RE: **The effect of oral rehydration solution and recommended home fluids on diarrhoea mortality**. *International Journal of Epidemiology* 2010, **39**:i75-i87.

58. Grandy G, Medina M, Soria R, Teran CG, Araya M: **Probiotics in the treatment of acute rotavirus diarrhoea. A randomized, double-blind, controlled trial using two different probiotic preparations in Bolivian children**. *BMC Infectious Diseases* 2010, **10**:DOI: 10.1186.

59. Teran CG, Teran-Escalera CN, Villarroel P: **Nitazoxanide vs. probiotics for the treatment of acute rotavirus diarroea in children: a randomized, single-blind, controlled trial in Bolivian children**. *International Journal of infectious Diseases* 2009, **13**:518-523.

60. Bagchi P, Nandi S, Chattopadhyay S, Bhowmick R, Halder UC, Nayak MK, Kobayashi N, Chawla-Sarkar M: **Identification of common human host genes involved in pathogenesis of different rotavirus strains: an attempt to recognize probable antiviral targets**. *Virus Research* 2012, **169**:144-153.

61. He H, Zhou D, Fan W, Fu X, Zhang J, Shen Z, Li J, Li J, Wu Y: **Cyclophilin A inhibits rotavirus replication by facilitating host interferon type-I production**. *Biochemical and Biophysical Research Communications* 2012, **422**:664-669.

62. Kim Y, George D, Prior AM, Prasain K, Hao S, Le DD, Hua DH, Chang KO: **Novel triacsin C analogs as potential antivirals against rotavirus infections**. *European Journal of Medicinal Chemistry* 2012, **50**:311-318.

63. Guererero CA, Murillo A, Acosta O: **Inhibition of rotavirus infection in cultured cells by N-acetyl-cysteine, PPARϒ agonists and NSAIDs**. *Antiviral Research* 2012, **96**:1-12.

64. Hardy ME, Hendricks JM, Paulson JM, Faunce NR: **18-Beta-glycyrrhetinic acid inhibits rotavirus replication in culture**. *Virology Journal* 2012, **9**:1-7.

65. Oh HM, Lee SW, Park MH, Kim MH, Ryu YB, Kim MS, Kim HH, Park KH, Lee WS, Park SJ, et al.: **Norkurarinol inhibits toll-like receptor 3 (TLR3)-mediated pro-inflammatory signaling pathway and rotavirus replication**. *Journal of Pharmacological Sciences* 2012, **118**:161-170.

66. Dennehy PH: **Rotavirus vaccines: an overview**. *Clinical Microbiology Reviews* 2008, **21**:198-208.

67. Vesikari T: **Rotavirus vaccination: a concise review**. *Clinical Microbiology and Infection* 2012, **18**:57-63.

68. Vesikari T, Joensuu J: **Review of rotavirus vaccine trials in Finland**. *Journal of Infectious Diseases* 1996, **174**:S81-S87.

69. Ward RL, McNeal MM, Steele AD: **Why does the world need another rotavirus vaccine?** *Therapeutics and Clinical Risk Management* 2008, **4**:49-63.

70. Glass R, Parashar UD, Bresse J, Turcios R, Fischer TK, Widdowson MA, Jiang B, Gentsch JR: **Rotavirus vaccines: current prospects and future challenges**. *Lancet* 2006, **368**:323-332.

71. Mirzayeva R, Steele AD, Parashar UD, Zaman K, Neuzil KM, Nelsong EAS: **Evaluation of rotavirus vaccines in Asia-are there lessons to be learnt?** *Vaccine* 2009, **27**:F120-F129.

72. Patel M, Shane AL, Parashar UD, Jiang B, Gentsch JR, Glass RI: **Oral rotavirus vaccines: how well will they work where they are needed most?** *Journal of Infectious Diseases* 2009, **200**:S39-S48.

73. Desai R, Curns AT, Patel MM, Parashar UD: **Trends in intussusception-associated deaths among US infants from 1979-2007**. *Journal of Pediatrics* 2012, **160**:456-460.

74. Gilliland SM, Forrest L, Carre H, Jenkins A, Berry N, Martin J, Minor P, Schepelmann S: **Investigation of porcine circovirus contamination in human vaccines**. *Biologicals* 2012, **40**:270-277.

75. Madsen LB, Ustrup M, Fischer TK, Bygbjerg IC, Konradsen F: **Reduced price on rotavirus vaccines: enough to facilitate access where most needed?** *Bulletin of the World Health Organization* 2012, **90**:554-556.

76. Weycker D, Sofrygin O, Kemner JE, Pelton SI, Oster G: **Cost of routine immunization of young children against rotavirus infection with Rotarix versus RotaTeq**. *Vaccine* 2009, **27**:4930-4937.

77. Martella V, Banyai K, Matthijnssens J, Buonavoglia C, Ciarlet M: **Zoonotic aspects of rotaviruses**. *Veterinary Microbiology* 2010, **140**:246-255.

78. Fu C, He Q, Xu J, Xie H, Ding P, Hu W, Dong Z, Liu X, Wang M: **Effectiveness of the Lanzhou lamb rotavirus vaccine against gasteroenteritis among children**. *Vaccine* 2012, **31**:154-158.

79. Zade JK, Kulkarni PS, Desai SA, Sabale RN, Naik SP, Dhere RM: **Bovine rotavirus pentavalent vaccine development in India**. *Vaccine* 2014, **32**:A124-A128.

80. Appaiahgari MB, Glass R, Singh S, Taneja S, Rongsen-Chandola T, Bhandari N, Mishra S, Vrati S: **Transplacental rotavirus IgG interferes with immune response to live oral rotavirus vaccine ORV-116E in Indian infants**. *Vaccine* 2014, **32**:651-656.

81. Bhandari N, Rongsen-Chandola T, Bavdekar A, John J, Antony K, Taneja S, Goyal N, Kawade A, Kang G, Singh Rathore S, et al.: **Efficacy of a monovalent human-bovine (116E) rotavirus vaccine in Indian children in the second year of life**. *Vaccine* 2014, **32S**:A110-A116.

82. Bhandari N, Rongsen-Chandola T, Bavdekar A, John J, Antony K, Taneja S, Goyal N, Kawade A, Kang G, Singh Rathore S, et al.: **Efficacy of a monovalent human-bovine (116E) rotavirus vaccine in Indian infants: a randomised, double-blind, placebo-controlled trial**. *Lancet* 2014, **383**:2136-2143.

83. Jiang B, Gentsch JR, Glass RI: **Inactivated rotavirus vaccines: a priority for accelerated vaccine development**. *Vaccine* 2008, **26**:6754-6758.

84. Wang Y, Azevedo M, Saif LJ, Gentsch JR, Glass RI, Jiang B: **Inactivated rotavirus vaccine induces protective immunity in gnotobiotic piglets**. *Vaccine* 2010, **28**:5432-5436.

85. Johansen K, Schroder U, Svensson L: **Immunogenecity and protective efficacy of a formalin-inactivated rotavirus vaccine combined with lipid adjuvants**. *Vaccine* 2003, **21**:368-375.

86. Moyle P, Toth I: **Modern subunit vaccines: development, components, and research opportunities**. *Pharmaceutical and Medicinal Chemistry* 2013, **8**:360-376.

87. Johansen K, Hinkula J, Espinoza F, Levi M, Zeng C, Ruden U, Vesikari T, Mary Estes M, Svensson L: **Humoral and cell-mediated immune responses in humans to the NSP4 enterotoxin of rotavirus**. *Journal of Medical Virology* 1999, **59**:369-377.

88. McNeal MM, Basu M, Bean JA, Clements JD, Choi AHC, Ward RL: **Identification of an immunodominant $CD4^+$ T cell epitope in the VP6 protein of rotavirus following intranasal immunization of BALB/c mice**. *Virology* 2007, **363**:410-418.

89. Choi AHC, McNeal MM, Basu M, Bean JA, VanCott JL, Clements JD, Ward RL: **Functional mapping of protective epitopes within the rotavirus VP6 protein in mice belonging to different haplotypes**. *Vaccine* 2003, **21**:761-767.

90. Zhao W, Pahar B, Sestak K: **Identification of rotavirus VP6-specific CD4$^+$ T cell epitopes in a G1P[8] human rotavirus infected rhesus macaque**. *Virology* 2008, **3**:9-15.

91. Wei J, Li J-T, Zhang X-P, Tang Y, Wang J-X, Zhang B, Wu Y-Z: **Identification of an HLA-A*0201-restricted cytotoxic T-lymphocyte epitope in rotavirus VP6 protein**. *Journal of General Virology* 2006, **87**:3393-3396.

92. Banos DM, Lopez S, Arias CF, Esquivel FR: **Identification of T-helper cell epitope on the rotavirus VP6 protein**. *Journal of Virology* 1997, **71**:419-426.

93. Choi AHC, Basu M, McNeal MM, Flint J, VanCott JL, Clements JD, Ward RL: **Functional mapping of protective domains and epitopes in the rotavirus VP6 protein**. *Journal of Virology* 2000, **74**:11574-11580.

94. Jaimes MC, Feng N, Greenberg HB: **Characterization of homologous and hetrologous rotavirus-specific T-cell responses in infants and adult mice**. *Journal of Virology* 2005, **79**:4568-4579.

95. Choi AH, McNeal MM, Basu M, Flint JA, Stone SC, Clements JD, Bean JA, Poe SA, VanCott JL, Ward RL: **Intranasal or oral immunization of inbred and outbred mice with murine or human rotavirus VP6 proteins protects against viral shedding after challenge with murine rotaviruses**. *Vaccine* 2002, **20**:3310-3321.

96. Choi AH, McNeal MM, Flint JA, Basu M, Lycke NY, Clements JD, Bean JA, Davis HL, McCluskie MJ, VanCott JL, et al.: **The level of protection against rotavirus shedding in mice following immunization with a chimeric VP6 protein is dependent on the route and the coadministered adjuvant**. *Vaccine* 2002, **20**:1733-1740.

97. Choi AHC, Basu M, McNeal MM, Bean JA, Clements JD, Ward RL: **Intranasal administration of an *Escherichia coli*-expressed codon-optimized rotavirus VP6 protein induces protection in mice**. *Protein Expression and Purification* 2004, **38**:205-216.

98. Dong JL, Liang BG, Jin YS, Zhang WJ, Wang T: **Oral immunization with pBsVP6-transgenic alfalfa protects mice against rotavirus infection**. *Virology* 2005, **339**:153-163.

99. Zhou B, Zhang Y, Wang X, Dong J, Wang B, Han C, Yu J, Li D: **Oral administration of plant-based rotavirus VP6 induces antigen-specific IgAs, IgGs and passive protection in mice**. *Vaccine* 2010, **28** 6021–6027.

100. Soler E, Parez N, Passet B, Dubuquoy C, Riffault S, Pillot M, Houdebine LM, Schwartz-Cornil I: **Recombinant rotavirus inner core proteins produced in the milk of transgenic rabbits confer a high level of protection after intrarectal delivery**. *Vaccine* 2007, **25**:6373-6380.

101. Blutt SE, Warfield KL, Estes MK, Conner ME: **Differential requirements for T cells in virus-like particle- and rotavirus-induced protective immunity**. *Journal of Virology* 2008, **82**:3135-3138.

102. Feng NG, Lawton JA, Gilbert J, Kuklin N, Vo P, Prasad BVV, Greenberg HB: **Inhibition of rotavirus replication by a non-neutralizing rotaviras VP6-specific IgA mAb**. *Journal of Clinical Investigation* 2002, **109**:1203-1213.

103. VanCott JL, Prada AE, McNeal MM, Stone SC, Basu M, Huffer B, Smiley KL, Shao MY, Bean JA, Clements JD, et al.: **Mice develop effective but delayed protective immune responses when immunized as neonates either intranasally with non-living VP6/LT(R192G) or orally with live rhesus rotavirus vaccine candidates**. *Journal of Virology* 2006, **80**:4949-4961.

104. Lin SL, Tian P: **Detailed computational analysis of a comprehensive set of group A rotavirus NSP4 proteins**. *Virus Genes* 2003, **26**:271-282.

105. Hyser JM, Zeng CQY, Beharry Z, Palzkill T, Estes MK: **Epitope mapping and use of epitope-specific antisera to characterize the VP5* binding site in rotavirus SA11 NSP4**. *Virology* 2008, **373**:211-228.

106. Kavanagh OV, Ajami NJ, Cheng E, Ciarlet M, Guerrero RA, Zeng CQY, Crawford SE, Estes MK: **Rotavirus enterotoxin NSP4 has mucosal adjuvant properties**. *Vaccine* 2010, **28**:3106-3111.

107. Kim TG, Befus N, Langridge WHR: **Co-immunization with an HIV-1 Tat transduction peptide-rotavirus enterotoxin fusion protein stimulates a Th1 mucosal immune response in mice**. *Vaccine* 2004, **22**:431-438.

108. Yu J, Langridge WHR: **A plant-based multicomponent vaccine protects mice from enteric diseases**. *Nature Biotechnology* 2001, **19**:548-552.

109. Andersson AMC, Hakansson KO, Jensen BAH, Christensen D, Andersen P, Thomsen AR, Christensen JP: **Increased immunogenicity and protective efficacy of influenza M2e fused to a tetramerizing protein**. *PloS One* 2012, **7**:e46395.

110. Ghosh A, Chattopadhyay S, Chawla-Sarkar M, Nandy P, Nandy A: **In silico study of rotavirus VP7 surface accessible conserved regions for antiviral drug/vaccine design**. *PloS One* 2012, **7**:e40749.

111. Favacho ARM, Kurtenbach E, Sardi SI, Gouvea VS: **Cloning, expression, and purification of recombinant bovine rotavirus hemagglutinin, VP8*, in *Escherichia coli***. *Protein Expression and Purification* 2006, **46**:196-203.

112. Xie L, Yan M, Wanga X, Yea J, Mia K, Yana S, Niua X, Lia H, Sun M: **Immunogenicity and efficacy in mice of an adenovirus-based bicistronic rotavirus vaccine expressing NSP4 and VP7**. *Virus Research* 2015, **210**:298-307.

113. Xue M, Yu L, Che Y, Lin H, Zeng Y, Fang M, Li T, Ge S, Xia N: **Characterization and protective efficacy in an animal model of a novel truncated rotavirus VP8 subunit parenteral vaccine candidate**. *Vaccine* 2015, **33**:2606-2613.

114. Kovacs-Nolan J, Mine Y: **Tandem copies of a human rotavirus VP8 epitope can induce specific neutralizing antibodies in BALB/c mice**. *Biochimica et Biophysica Acta* 2006, **1760**:1884-1893.

115. Clarke E, Desselberger U: **Correlates of protection against human rotavirus disease and the factors influencing protection in low-income settings**. *Mucosal Immunology* 2015, **8**:1-17.

116. Hu L, Crawford SE, Czako R, Cortes-Penfield NW, Smith DF, Le Pendu J, Estes MK, Prasad BVV: **Cell attachement protein VP8* of a human rotavirus specifically interacts with A-type histo-blood group antigen**. *Nature* 2012, **485**:256-259.

117. Stencel-Baerenwald JE, Reiss K, Reiter DM, Stehle T, Dermody TS: **The sweet spot: defining virus-sialic acid interactions**. *Nature Reviews Microbiolgy* 2014, **12**:739-749.

118. Lentza EM, Mozgovojb MV, Bellidob D, Dus Santosb MJ, Wigdorovitzb A, Bravo-Almonacida FF: **VP8* antigen produced in tobacco transplastomic plants confers protection against bovine rotavirus infection in a suckling mouse model**. *Journal of Biotechnology* 2011, **156**:100-107.

119. Marelli B, Rosa Perez A, Banchio C, de Mendoza D, Magni C: **Oral immunization with live *Lactococcus lactis* expressing rotavirus VP8*subunit induces specific immune response in mice**. *Journal of Virological Methods* 2011, **175**:28-37.

120. Kovacs-Nolan J, Sasaki E, Yoo DW, Mine Y: **Cloning and expression of human rotavirus spike protein, VP8*, in *Escherichia coli***. *Biochemical and Biophysical Research Communications* 2001, **282**:1183-1188.

121. Andres I, Rodriguez-Diaz J, Buesa J, Zueco J: **Yeast expression of the VP8*fragment of the rotavirus spike protein and its use as immunogen in mice**. *Biotechnology and Bioengineering* 2006, **93**:89-98.

122. Wen X, Cao D, Jones RW, Li J, Szu S, Hoshino Y: **Construction and characterization of human rotavirus recombinant VP8* subunit parenteral vaccine candidates**. *Vaccine* 2012, **30**:6121-6126.

123. Wen X, Wen K, Cao D, Li G, Jones RW, Li J, Szu S, Hoshino Y, Yuan L: **Inclusion of a universal tetanus toxoid $CD4^+$ T cell epitope P2 significantly enhanced the**

immunogenecity of recombinant rotavirus VP8* subunit parenteral vaccines. *Vaccine* 2014, **32**:4420-4427.

124. Lua LHL, Connors NK, Sainsbury F, Chuan YP, Wibowo N, Middelberg APJ: **Bioengineering virus like-particles as vaccines**. *Biotechnollogy and Bioengineering* 2014, **111**:425-440.

125. Zeltins A: **Constructions and characterizations of virus-like particles: a review**. *Molecular Biotechnology* 2012, **53**:92-107.

126. Roldao A, Mellado M, Castilho L, Carrondo M, Alves P: **Virus-like particles in vaccine development**. *Expert Review Vaccines* 2010, **9**:1149-1176.

127. Azevedo M, Viasova A, Saif L: **Human rotavirus virus-like particle vaccines evaluated in a neonatal gnotobiotic pig model of human rotavirus disease.** *Expert Review Vaccines* 2013, **12**:169-181.

128. El-Attar L, Oliver SL, Mackie A, Charpilienne A, Poncet D, Cohen J, Bridger JC: **Comparison of the efficacy of rotavirus VLP vaccines to a live homologous rotavirus vaccine in a pig model of rotavirus disease**. *Vaccine* 2009, **27**:3201-3208.

129. Palomares LA, Mena JA, Ramirez OT: **Simeltaneous expression of recombinant proteins in the insect cell-baculovirus system: production of virus-like particles**. *Methods* 2012, **56**:389-395.

130. Rodriguez-Limas WA, Tyo KEJ, Nielsen J, Ramirez OT, Palomares LA: **Molecular and process design for rotavirus-like particle production in *Saccharomyces cerevisiae***. *Microbial Cell Factories* 2011, **10**:DOI: 10.1186.

131. Palomares LA, Ramirez OT: **Challenges for the production of virus-like particles in insect cells: the case of rotavirus-like particles**. *Biochemical Engineering Journal* 2009, **45**:158-167.

132. Castro-Acosta RM, Revilla AL, Ramirez OT, Palomares LA: **Separation and quantification of double- and triple-layered rotavirus-like particles by capilary zone electrophoresis**. *Electrophoresis* 2010, **31**:1376-1381.

133. Tan M, Huang P, Xia M, Fang PA, Zhong W, McNeal M, Wei C, Jiang W, Jiang X: **Norovirus P particle, a novel platform for vaccine development and antibody production**. *Journal of Virology* 2011, **85**:753-764.

134. Tegerstedt K, Franzen AV, Andreasson K, Joneberg J, Heidari S, Ramqvist T, Dalianis T: **Murine polyomavirus virus-like particles as vectors for gene and immune therapy and vaccines against viral infections and cancer**. *Anticancer Research* 2005, **25**:2601-2608.

135. Liew MWO, Chuan YP, Middelberg APJ: **Reactive diafiltration for assembly and formulation of virus-like particles**. *Biochemical Engineering Journal* 2012, **68**:120-128.

136. Liew MWO, Rajendran A, Middelberg APJ: **Microbial production of virus-like particle vaccine protein at gram-per-litre levels**. *Journal of Biotechnology* 2010, **150**:224-231.

137. Liew MWO, Chuan YP, Middelberg APJ: **High-yield and scalable cell-free assembly of virus-like particles by dilution**. *Biochemical Engineering Journal* 2012, **67**:88-96.

138. Middelberg APJ, Rivera-Hernandez T, Wibowo N, Lua LHL, Fan Y, Magor G, Chang C, Chuan YP, Good MF, Batzloff MR: **A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines**. *Vaccine* 2011, **29**:7154-7162.

139. Chuan YP, Lua LHL, Middelberg APJ: **High-level expression of soluble viral structural protein in *Escherichia coli***. *Journal of Biotechnology* 2008, **134**:64-71.

140. Wibowo N, Chuan YP, Lua LHL, Middelberg APJ: **Modular engineering of a microbially-produced viral capsomere vaccine for influenza**. *Chemical Engineering Science* 2012, **103**:12-20.

141. Wibowo N, Wu Y, Fan Y, Meers J, Lua LHL, Middelberg APJ: **Non-chromatographic preparation of a bacterially produced single-shot modular virus-like particle capsomere vaccinefor avian influenza**. *Vaccine* 2015, **33**:5960-5965.

142. Krumbholz A, Bininda-Emonds ORP, Wutzler P, Zell R: **Phylogenetics, evolution and medical importance of polyomaviruses**. *Infection Genetics and Evolution* 2009, **9**:784-799.

143. Carbone M, Reale A, Sauro AD, Sthandier O, Garcia M-I, Maione R, Caiafa P, Amati P: **PARP-1 interaction with VP1 capsid protein regulates polyomavirus early gene expression**. *Journal of Molecular Biology* 2006, **363**:773-785.

144. Burton KS, Consigli RA: **Methylation of the polyomavirus major capsid protein VP1**. *Virus Research* 1996, **40**:141-147.

145. Neugebauer M, Walders B, Brinkman M, Ruehland C, Schumacher T, Bertling WM, Geuther E, Reiser COA, Reichel C, Strich S, et al.: **Development of a vaccine marker technology: display of B cell epitopes on the surface of recombinant polyomavirus-like pentamers and capsoids induces pepptide-specific antibodies in piglets after vaccination** *Biotechnology Journal* 2006, **1**:1435-1446.

146. Gillock ET, Rottinghaus S, Chang D, Cai X, Smiley SA, An K, Consigli RA: **Polyomavirus major capsid protein VP1 is capable of packaging cellular DNA when expressed in the baculovirus system**. *Journal of Virology* 1997, **71**:2857-2865.

147. Georgens C, Weyermann J, Zimmer A: **Recombinant virus like particles as drug delivery system**. *Current Pharmaceutical Biotechnology* 2005, **6**:49-55.

148. Cerqueira C, Schelhaas M: **Principles of polyoma- and papillomavirus uncoating**. *Medical Microbiology and Immunology* 2012, **201**:427-436.

149. Gedivilaite A, Dorn DC, Sasnauskas K, Pecher G, Bulavaite A, Lawatscheck R, Staniulis J, Dalianis T, Ramqvist T, Schonrich G, et al.: **VLPs derived from major capsid protein VP1 of different polyomaviruses differ in their ability to induce maturation in human dendritic cells**. *Virology* 2006, **354**:252-260.

150. Chuan YP, Fan Y, Lua LHL, Middelberg APJ: **Quantitative analysis of virus-like particle size and distribution by field-flow fractionation**. *Biotechnology and Bioengineering* 2008, **99**:1425-1433.

151. Noad R, Roy P: **Virus-like particles as immunogens**. *Trends Microbiology* 2003, **11**:438-444.

152. Caparros-Wanderley W, Clark B, Griffin BE: **Effect of dose and long-term storage on the immunogenicity of murine polyomavirus VP1 virus-like particles**. *Vaccine* 2004, **22**:352-361.

153. Rollman E, Ramqvist T, Zuber B, Tegerstedt K, Zuber AK, Klingstrom J, Eriksson L, Ljungberg K, Hinkula J, Wahren B, et al.: **Genetic immunization is augmented by murine polyomavirus VP1 pseudocapsids**. *Vaccine* 2003, **21**:2263-2267.

154. Stehle T, Harrison SC: **High-resolution structure of a polyomavirus VP1-oligosaccharide complex: implications for assembly and receptor binding**. *European Molecular Biology Organization Journal* 1997, **16**:5139-5148.

155. Rivera-Hernandez T, Hartas J, Wu Y, Chuan YP, Lua LHL, Good M, Batzloff MR, Middelberg APJ: **Self-adjuvanting modular virus-like particles for mucosal vaccination against group A streptococcus (GAS)**. *Vaccine* 2013, **31**:1950-1955.

156. Kushnir N, Streatfield SJ, Yusibov V: **Virus-like particles as a highly efficient vaccine platform: Diversity of targets and production systems and advances in clinical development**. *Vaccine* 2012, **31**:58-83.

157. Teunissen EA, de Raad M, Mastrobattista E: **Production and biomedical applications of virus-like particles derived from polyomaviruses**. *Journal of Controlled Release* 2013, **172**:305-321.

158. Chuan YP, Rivera-Hernandez T, Wibowo N, Connors NK, Wu Y, Hughes FK, Lua LHL, Middelberg APJ: **Effects of pre-existing anti-carrier immunity and antigenic element multiplicity on efficacy of a modular virus-like particle vaccine**. *Biotechnology and Bioengineering* 2013, **110**:2343-2351.

159. Wibowo N, Hughes FK, Fairmaid EJ, Lua LHL, Brown LE, Middelberg APJ: **Protective efficacy of a bacterially produced modular capsomere presenting M2e from influenza: extending the potential of broadly cross-protecting epitopes**. *Vaccine* 2014, **32**:3651-3655.

160. Graziano G, Merlino A: **Molecular bases of protein halotolerance**. *Biochimica et Biophysica Acta* 2014, **1844**:850-858.

161. Khrustalev VV, Eugene VB: **Stabilization of secondary structure elements by specific combinations of hydrophilic and hydrophobic amino acid residues is more important for proteins encoded by GC-poor genes**. *Biochimie* 2012, **94**:2706-2715.

162. Lua LHL, Fan Y, Chang C, Connors NK, Middelberg APJ: **Synthetic biology design to display an 18 kDa rotavirus large antigen on a modular virus-like particle** *Vaccine* 2015, **33**:5937-5944.

163. Mann DF, Shah K, Stein D, Snead GA: **Protein hydrophobicity and stability support the thermodynamic theory of protein degradation**. *Biochimica et Biophysica Acta* 1984, **788**:17-22.

164. Pace CN, Fu H, Fryar KL, Landua J, Trevino SR, Shirley BA, Hendricks MM, Iimura S, Gajiwala K, Scholtz JM, et al.: **Contribution of hydrophobic interactions to protein stability**. *Journal of Molecular Biology* 2011, **408**:514-528.

165. Ajmera A, Scherließ R: **Stabilisation of proteins via mixtures of amino acids during spray drying**. *International Journal of Pharmaceutics* 2014, **463**:98-107.

166. Papaneophytou CP, Kontopidis G: **Statistical approaches to maximize recombinant protein expression in *Escherichia coli*: a general review**. *Protein Expression and Purification* 2014, **94**:22-32.

167. Ciccaglione A, Marcantonio C, Costantino A, Equestre M, Geraci A, Rapicetta M: **Expression and membrane association of hepatitis C virus envelope 1 protein**. *Virus Genes* 2000, **21**:223-226.

168. Hwang PM, Pan JS, Sykes BD: **Targeted expression, purification, and cleavage of fusion proteins from inclusion bodies in *Escherichia coli***. *FEBS Letters* 2014, **588**:247-252.

169. Idicula-Thomas S, Balaji PV: **Understanding the relationship between the primary structure of proteins and its propensity to be soluble on overexpression in *Escherichia coli***. *Protein Sci* 2004, **14**:582-592.

170. Singh SM, Panda AK: **Solubilization and refolding of bacterial inclusion body proteins**. *Journal of Bioscience and Bioengineering* 2005, **99**:303-310.

171. Studier FW: **Protein production by auto-induction in high-density shaking cultures**. *Protein Expression and Purification* 2005, **41**:207-234.

172. Prasad S, Khadatare PB, Roy I: **Effect of chemical chaperones in improving the solubility of recombinant proteins in *Escherichia coli***. *Applied Environmental Microbiology* 2011, **77**:4603-4609.

173. Ghosh S, Rasheedi S, Rahim SS, Banerjee S, Choudhary RK, Chakhaiyar P, Ehtesham NZ, Mukhopadhyay S, Hasnain SE: **Method for enhancing solubility of the expressed recombinant proteins in *Escherichia coli***. *BioTechniques* 2004, **37**:418-423.

174. Vernet E, Kotzsch A, Voldborg B, Sundsstrom M: **Screening of genetic parameters for soluble protein expression in *Escherichia coli***. *Protein Expression and Purification* 2011, **77**:104-111.

175. Niiranen L, Espelid S, Karlsen CR, Mustonen M, Paulsen SM, Heikinheimo P, Willassen NP: **Comparative expression study to increase the solubility of cold adapted *Vibrio* proteins in *Escherichia coli***. *Protein Expression Purification* 2007, **52**:210-218.

176. Costa S, Almeida A, Castro A, Lucilia D: **Fusion tags for protein solubility, purification and immunogenecity in *Escherichia coli*: the novel Fh8 system**. *Frontiers in Microbiology* 2014, **5**:1-20.

177. Connors NK, Wu Y, Lua LHL, Middelberg APJ: **Improved fusion tag cleavage strategies in the downstreamprocessing of self-assembling virus-like particle vaccines**. *Food and Bioproducts Processing* 2014, **92**:143-151.

178. Tekewe A, Connors NK, Sainsbury F, Wibowo N, Lua LHL, Middelberg APJ: **A rapid and simple screening method to identify conditions for enhanced stability of modular vaccine candidates**. *Biochemical Engineering Journal* 2015, **100**:50-58.

179. Wang W, Nema S, Teagarden D: **Protein aggregation-pathways and influencing factors**. *International Journal of Pharmaceutics* 2010, **390**:89-99.

180. Chi EY, Krishnan S, Randolph TW, Carpenter JF: **Physical stability of proteins in aqueous solution: mechanism and driving forces in non-native protein aggregation**. *Pharmaceutical Research* 2004, **20**:1325-1336.

181. Golovanov AP, Hautbergue GM, Wilson SA, Lian LY: **A simple method for improving protein solubility and long-term stability**. *Journal of American Chemical Society* 2004, **126**:8933-8939.

182. Lee HJ, McAuley A, Schilke KF, McGuire J: **Molecular origins of surfactant-mediated stabilization of protein drugs**. *Advanced Drug Delivery Reviews* 2011, **63**:1160-1171.

183. Matsuoka T, Tomita S, Hamada H, Shiraki K: **Amidated amino acids are prominent additives for preventing heat-induced aggregation of lysozyme**. *Journal of Bioscience and Bioengineering* 2007, **103**:440-443.

184. Leibly DJ, Nguyen TN, Kao LT, Hewitt SN, Barrett LK, Van Voorhis WC: **Stabilizing additives added during cell lysis aid in the solubilization of recombinant proteins**. *PloS One* 2012, **7**:e52482.

185. Hamada H, Shiraki K: **L-Argininamide improves the refolding more effectively than L-arginine**. *Journal of Biotechnology* 2007, **130**:153-160.

186. Kumar N, Kishore N: **Structure and effect of sarcosine on water and urea by using molecular dynamics simulations: implications in protein stabilization**. *Biophysical Chemistry* 2013, **171**:9-15.

187. Kumar N, Kishore N: **Protein stabilization and counteraction of denaturing effect of urea by glycine betaine**. *Biophysical Chemistry* 2014, **189**:16-24.

188. Mohr J, Chuan YP, Lua LHL, Middelberg APJ: **Virus-like particle formulation optimization by miniaturized high-throughput screening**. *Methods* 2013, **60**:248-256.

189. Tsumoto K, Umetsu M, Kumagai I, Ejima D, Philo JS, Arakawa T: **Role of arginine in protein refolding, solubilization and purification**. *Biotechnology Progress* 2004, **20**:1301-1308.

190. Demeule B, Gurny R, Arvinte T: **Detection and characterization of protein aggregates by fluorescence microscopy**. *International Journal of Pharmaceutics* 2007, **329**:37-45.

191. Demeule B, Lawrence MJ, Drake AF, Gurny R, Arvinte T: **Characterization of protein aggregation: the case of a therapeutic immunoglobulin**. *Biochimica et Biophysica Acta* 2007, **1774**:146-153.

192. Bondos SE, Bicknell A: **Detection and prevention of protein aggregation before, during and after purification**. *Analytical Biochemistry* 2003, **316**:223-231.

193. Gutmann DAP, MizohataI E, Newstead S, Ferrandon S, Henderson PJF, Van Veen HW, Byrne B: **A high-throughput method for membrane protein solubility screening: the ultracentrifugation dispersity sedimentation assay**. *Protein Science* 2007, **16**:1422-1428.

194. Vincentelli R, Canaan S, Campanacci V, Valencia C, Maurin D, Frassinetti F, Scappucini-Calvo, Bourne Y, Cambillau C, Bignon C: **High-throughput automated refolding screening of inclusion bodies**. *Protein Science* 2004, **13**:2782-2792.

195. Berg A, Schuetz M, Dismer F, Hubbuch J: **Automated measurement of apparent protein solubility to rapidly assess complex parameter interactions**. *Food and Bioproducts Processing* 2014, **92**:133-142.

196. Dasnoy S, Dezutter N, Lemoine D, Le Bras V, Preat V: **High-throughput screening of excipients intended to prevent antigen aggregation at air-liquid interface**. *Pharmaceutical Research* 2011, **28**:1591-1605.

197. Wiendahl M, Volker C, Husemann I, Krarupa J, Staby A, Scholl S, Hubbuch J: **A novel method to evaluate protein solubility using a high-throughput screening approach**. *Chemical Engineering Science* 2009, **64**:3778-3788.

198. Hanke AT, Ottens M: **Purifying biopharmaceuticals: knowledge-based chromatographic process development**. *Trends in Biotechnology* 2014, **32**:210-220.

199. Yang H, Yang H-m, Kong P, Zhu Y-m, Dai S-g, Zheng G: **Concentration measurement of particles by number fluctuation in dynamic light back scattering**. *Powder Technology* 2013, **246**:499-503.

200. Li Z, Wang Y, Shen J, Liu W, Sun X: **The measurement system of nanoparticle size distribution from dynamic light scatering data**. *Optics and Lasers in Engineering* 2014, **56**:94-98.

201. Rusu D, Genoe D, van Puyvelde P, Peuvrel-Disdier E, Navard P, Fuller GG: **Dynamic light scattering during shear: measurements of diffusion coefficients**. *Polymer* 1999, **40**:1353-1357.

202. Lynch SA, Gill RT: **Synthetic biology: new strategies for directing design**. *Metabolic Engineering* 2012, **14**:205-211.

203. Vohra P, Blakely GW: **Easing the global burden of diarrhoeal diseases: can synthetic biology help?** *Systems and Synthetic Biology* 2013, **7**:73-78.

204. Wagner JM, Alper HS: **Synthetic biology and molecular genetics in non-conventional yeasts: current tools and future advances**. *Fungal Genetics and Biology* 2015, **89**:126-136.

205. Chen X, Zaro JL, Shen WC: **Fusion protein linkers: property, design and functionality**. *Advanced Drug Delivery Reviews* 2013, **65**:1357-1369.

206. Zhao HL, Yao XQ, Xue C, Wang Y, Xiong XH, Liu ZM: **Increasing the homogeneity, stability and activity of human serum albumin and interferon-α2b fusion protein by linker engineering**. *Protein Expression and Purification* 2008, **61**:73-77.

207. Hao HJ, Jiang YQ, Zheng YL, Ma R, Yu DW: **Improved stability and yield of Fv targeted superantigen by introducing both linker and disulfide bond into the targeting moiety**. *Biochimie* 2005, **87**:661-667.

208. Klement M, Liu C, Loo BLW, Choo ABH, Siak-Wei Ow D, Lee DY: **Effect of linker flexibility and length on the functionality of a cytotoxic engineered antibody fragment**. *Journal of Biotechnology* 2015, **199**:90-97.

209. Trevino SR, Scholtz JM, Pace CN: **Amino acid contribution to protein stability: Asp, Glu and Ser contribute more favorably than the other hydrophilic amino acids in RNase Sa**. *Journal Molecular Biology* 2007, **366**:449-460.

210. Abidin RS, Lua LHL, Middelberg APJ, Sainsbury F: **Insert engineering and solubility screening improves recovery of virus-like particle subunits displaying hydrophobic epitopes**. *Protein Science* 2015, **24**:1820-1828.

211. Anggraeni MR: **Engineering of virus-like particles for alternative vaccine candidate targeting hyper-variable peptide antigen-element. PhD Thesis, The University of Queensland. DOI: 10.14264/uql.2015.135** 2014.

212. Stubenrauch K, Bachmann A, Rudolph R, Lilie H: **Purification of a viral coat protein by an engineered polyionic sequence**. *Journal of Chromatography B* 2000, **737**:77-84.

213. Zeng J, Zhang L, Li Y, Wang Y, Wang M, Duan X, He ZG: **Over-producing soluble protein complex and validating protein-protein interaction through a new bacterial co-expression system**. *Protein Expression and Purification* 2010, **69**:47-53.

214. Masutani M, Machida K, Kobayashi T, Yokoyama S, Imataka H: **Reconstitution of eukaryotic translation initiation factor 3 by co-expression of the subunits in a human cell-derived in vitro protein synthesis system**. *Protein Expression and Purification* 2013, **87**:5-10.

215. Kerrigan JJ, Xie Q, Ames RS, Lu Q: **Production of protein complexes via co-expression**. *Protein Expression and Purification* 2011, **75**:1-14.

216. Busso D, Peleg Y, Heidebrecht T, Romier C, Jacobovitch Y, Dantes A, Salim L, Troesch E, Schuetz A, Heinemann U, et al.: **Expression of protein complexes using multiple *Escherichia coli* protein co-expression systems: a benchmarking study**. *Journal of Structural Biology* 2011, **175**:159-170.

217. Karamitros CS, Konrad M: **Bacterial co-expression of the α and β protomers of human L-asparaginase-3: achieving essential N-terminal exposure of a catalytically critical threonine located in the β-subunit**. *Protein Expression and Purification* 2014, **93**:1-10.

218. Cheng CY, Yu YJ, Yang MT: **Co-expression of ω subunit in *Escherichia coli* is required for the maintenance of enzymatic activity of *Xanthomonas campestris* RNA polymerase**. *Protein Expression and Purification* 2010, **69**:91-98.

219. Diebold M-L, Fribourg S, Koch M, Metzger T, Romier C: **Deciphering correct strategies for multiprotein complex assembly by co-expression : application to complexes as large as the histone octamer**. *Journal of Structural Biology* 2011, **175**:178-188.

220. Tolia NH, Joshua-Tor L: **Strategies for protein co-expression in *Escherichia coli*** *Nature Methods* 2006, **3**:55-64.

221. Chen R: **Bacterial expression systems for recombinant protein production: *Escherichia coli* and beyond**. *Biotechnology Advances* 2012, **30**:1102-1107.

222. Sommer B, Friehs K, Flaschel E: **Efficient production of extracellular proteins with E. coli by means of optimized co-expression of bacteriocin release proteins**. *Journal of Biotechnology* 2010, **145**:350-358.

223. Sugase K, Landes MA, Wright PE, Martinez-Yamout M: **Overexpression of post-translationally modified peptides in *Escherichia coli* by co-expression with modifying enzymes**. *Protein Expression and Purification* 2008, **57**:108-115.

224. Chen Y, Song J, Sui SF, Wanga DN: **DnaK and DnaJ facilitated the folding process and reduced inclusion body formation of magnesium transporter CorA overexpressed in *Escherichia coli***. *Protein Expression and Purification* 2003, **32**:221-231.

225. Kohda J, Endo Y, Okumura N, Kurokawa Y, Nishihara K, Yanagi H, Yura T, Fukuda H, Kondo A: **Improvement of productivity of active form of glutamate racemase in *Escherichia coli* by co-expression of folding accessory proteins**. *Biochemical Engineering Journal* 2002, **10**:39-45.

226. Kondo A, Kohda J, Endo Y, Shiromizu T, Kurokawa Y, Nishihara K, Yanagi H, Yura T, Fukuda H: **Improvement of productivity of active horseradish peroxidase in *Escherichia coli* by co-expression of Dsb proteins**. *Journal of Bioscience and Bioengineering* 2000, **90**:600-606.

227. Jhamb K, Sahoo DK: **Production of soluble recombinant proteins in *Escherichia coli*: effects of process conditions and chaperone co-expression on cell growth and production of xylanase**. *Bioresource Technology* 2012, **123**:135-143.

228. Hsu SY, Lin YS, Li SJ, Lee WC: **Co-expression of a heat shock transcription factor to improve conformational quality of recombinant protein in *Escherichia coli***. *Journal of Bioscience and Bioengineering* 2014, **118**:242-248.

229. Stols L, Zhou M, Eschenfeldt WH, Millard CS, Abdullah J, Collart FR, Kim Y, Donnelly MI: **New vectors for co-expression of proteins: structure of Bacillus subtilis ScoAB obtained by high-throughput protocols**. *Protein Expression and Purification* 2007, **53**:396-403.

230. Men Y, Zhu Y, Zeng Y, Izumori K, Sun Y, Ma Y: **Co-expression of D-glucose isomerase and D-psicose 3-epimerase: development of an efficient one-step production of D-psicose**. *Enzyme and Microbial Technology* 2014, **65**:1-5.

231. Jiang M, Chen X, Liang L, Liu R, Wan Q, Wu M, Zhang H, Ma J, Chen K, Ouyang P: **Co-expression of phosphoenolpyruvate carboxykinase and nicotinic acid phosphoribosyltransferase for succinate production in engineered *Escherichia coli***. *Enzyme and Microbial Technology* 2014, **56**:8-14.

232. Wakamori M, Umehara T, Yokoyama S: **A series of bacterial co-expression vectors with rare-cutter recognition sequences**. *Protein Expression and Purification* 2010, **74**:88-98.

233. Thakur KG, Jaiswal RK, Shukla JK, Praveena T, Gopal B: **Over-expression and purification strategies for recombinant multi-protein oligomers: a case study of Mycobacterium**

tuberclosis δ/anti-δ factor protein complexes. *Protein Expression and Purification* 2010, **74**:223-230.

234. Chuan YP, Wibowo N, Lua LHL, Middelberg APJ: **The economics of virus-like particles and capsomere vaccines**. *Biochemical Engineering Journal* 2014, **90**:255-263.

235. Hudgens MG, Gilbert PB, Self SG: **Endpoints in vaccine trials**. *Statistical Methods in Medical Research* 2004, **13**:1-26.

236. Yang X, Yuan L: **Neonatal gnotobiotic pig models for studying viral pathogenesis, immune response and for vaccine evaluation**. *British Journal of Virology* 2014, **1**:87-91.

237. Ward RL, McNeal MM, Sheridan JF: **Development of an adult mouse model for studies on protection against rotavirus**. *Journal of Virology* 1990, **64**:5070-5075.

238. Guenet J, Bonhomme F: **Wild mice: an ever-increasing contribution to a popular mammalian model**. *Trends in Genetics* 2003, **19**:24-31.

239. Buragohain M, Dhale GS, Raut C, Kang G, Chitambar SD: **Analyses of clinical, pathological and virological features of human rotavirus strain, YO induced gastroenteritis in infants BALB/c mice**. *Microbes and Infection* 2011, **13**:331-338.

240. Blutt SE, Warfield KL, O`Neal C, Estes MK, Conner ME: **Host, viral, and vaccine factors that determine protective efficacy induced by rotavirus and virus-like particles**. *Vaccine* 2006, **24**:1170-1179.

241. Nezlin R, Mozes E: **Detection of antigens in immune complexes by a dot blot assay**. *Journal of Immunological Methods* 1995, **184**:273-276.

242. Bishai FR, Galli R: **Enzyme-linked immunosorbent assay for detection of antibodies to influenza A and B and parainfluenza type 1 in sera of patients**. *Journal of Clinical Microbiology* 1978, **8**:648-656.

243. Ciarlet M, Conner ME: **Evaluation of rotavirus vaccines in small animal models**. *Methods in Molecular Medicine* 2000, **34**:147-187.

244. Istrate C, Hinkula J, Charpilienne A, Poncet D, Cohen J, Svensson L, Johansen K: **Parenteral adminstration of RF 8-2/6/7 rotavirus-like particles in a one-dose regimen induce protective immunity in mice**. *Vaccine* 2008, **26**:4594-4601.

245. Gonzalez DD, Mozgovoj MV, Bellido D, Rodriguez DV, Fernandez FM, Wigdorovitiz A, Parreno VG, Santos MJD: **Evaluation of a bovine rotavirus VP6 vaccine efficacy in the calf model of infection and disease**. *Veterinary Immunology and Immunopathology* 2010, **137**:155-160.

246. McNeal MM, Stone SC, Basu M, Bean JA, Clements JD, Henderson BA, Choi AHC, Ward RL: **Protection against rotavirus shedding after intranasal immunization of mice with a chimeric VP6 protein does not require intestinal IgA**. *Virology* 2006, **346**:338-347.

247. El-Senousy WM, Shahein YE, Barakat AB, Ghanem HE, El-Hakim AE, Ameen SM: **Molecular cloning and immunogenecity evaluation of rotavirus structural proteins as candidate vaccine**. *International Journal of Biological Macromolecules* 2013, **59**:67-71.

Chapter 3

A rapid and simple screening method to identify conditions for enhanced stability of modular vaccine candidates

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A rapid and simple screening method to identify conditions for enhanced stability of modular vaccine candidates

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Abstract

Virus-like particles (VLPs) and capsomere subunits have been developed as safe and effective vaccine candidates in the fight against infectious diseases. These bioengineered structures are suitable platforms for repetitive high density presentation of foreign epitope modules. However, due to the module's diverse physicochemical properties, modularisation of capsomeres and VLPs requires tailoring of the physicochemical environment specific to each module to maintain protein stability against aggregation. Here we report a high-throughput technique for screening buffer components to stabilize capsomeres, based on light scattering analysis. This screening method was applied to modular capsomeres presenting peptide epitopes from the rotavirus spike protein VP8 subunit domain, engineered as a next-generation rotavirus vaccine candidate. Among various additives tested, non-ionic detergents, such as Triton X-100, Tween-80 and Tween-20, were able to stabilize modular capsomeres, either alone or in combination with L-arginine, as confirmed with high-resolution size exclusion chromatography. Results demonstrate that tailoring the nature of the environment surrounding self-assembling proteins using small organic molecules can enhance the bioprocessing of modular vaccine capsomeres. The developed screening method potentially provides a powerful approach for rapid tailoring of processing conditions specific to antigenic modules displayed on next-generation recombinant capsomere and VLP vaccines, for low-cost vaccine delivery at global scale.

Key words: virus-like particles, capsomeres, protein, aggregation, downstream processing, protein recovery.

3.1. Introduction

Virus-like particles (VLPs) are macromolecular assemblies of the viral structural protein(s) that retain antigenic features of the authentic virus without the viral genome [1-3]. VLPs have come into focus for their promising application in vaccination [4-7] because of their high safety and efficacy profile [8]; their repetitive and high density native display of epitopes leading to their self-adjuvanting property; their ability to present foreign epitopes on the surface; and their stability compared with soluble antigens [9-12]. So far, three VLP-based vaccines have been commercialized for human use against hepatitis B virus infection, human papillomavirus-induced cervical cancer and hepatitis E virus infection [4,13]. Numerous other VLP-based vaccines against many infectious agents have shown promising results under pre-clinical evaluation using small-animal models and in clinical trials [12,14-16]. Moreover, assembly-incompetent VLP sub-units, termed capsomeres, have recently gained attention for their potential as alternative low-cost second-generation vaccine candidates to VLPs [15,17-19]. Although most capsomeres showed less immunogenic response compared with the corresponding VLPs, their reduced efficacy was significantly compensated by using effective and safe adjuvants [20]. Some capsomeres could induce almost the same level of immune response as the corresponding VLPs when formulated with safe adjuvants [15,18,19].

Despite the significant benefits of the marketed VLP-based vaccines and the promising potential of those in the developmental pipeline, many will likely remain unaffordable for resource-poor countries due to their high manufacturing and processing costs from eukaryotic expression systems [7,17,21]. Minimizing production costs has been the focus of several developments to enable low-resource countries to use the final product with affordable cost [22]. The ability to manufacture VLPs from microbially-expressed sub-units via *in vitro* assembly in a cell-free reactor, under engineering controls, suggests huge potential for cost reduction [23]. Low-cost bacterial expression systems have been used for production of the VLP sub-units resulting in high yields [7,15,21,24,25]. For example, the murine polyomavirus major capsid protein, VP1, has been produced as a GST-VP1 fusion protein at gram-per-liter levels in *Escherichia coli (E. coli)* [7,21]. Enzyme-mediated cleavage of the GST tag followed by purification and separation by size exclusion chromatography (SEC) results in pure VP1 capsomeres that have been assembled *in vitro* to form VP1 VLPs in cell-free reactors [24,26]. The production of assembly-incompetent VP1 capsomeres using prokaryotic expression has also been reported [15,18]. Current research focuses on the modularization of foreign antigenic epitopes into VLPs or capsomeres to target specific diseases. Middelberg *et al.* [15] have developed VP1 VLPs that were able to display J8-peptide from the M-protein of Group A streptococcus; such antigen-loaded modular VLPs showed

protection in a mouse model following unadjuvanted nasal delivery [15,27]. The modular capsomeres containing single [15,18] or multiple modules [18] of influenza M2e peptide antigen gave protection in mice when formulated with Alhydrogel® [28].

The insertion of modules into the VLP subunits can be well tolerated, [15,18,27]; however results can be dependent on the physicochemical properties of modules. Thus, modularization may require tailoring of the processing conditions specific to each module to maintain protein colloidal stability. The stability of proteins is a major concern, particularly for the field of vaccination, where lower efficacy can be a result of poor vaccine stability [29]. A number of approaches for stabilization of proteins at different stages of processing are described in the literature [30,31]. Among those the simplest and the most common method is tailoring the environment surrounding the proteins [31]. This can be achieved by optimizing solution conditions such as pH, ionic strength [31,32] or by adding stabilizing additives [32]. Several additives [29,33-40] have been studied for their potential to stabilize proteins at different stages of processing, formulation or upon storage.

Despite the availability of various stabilizing additives, identification of the most effective additive from a large experimental space for each target protein is laborious and time-consuming using analytical methods with low-throughput capacity [41,42]. Therefore, the use of high-throughput screening (HTS) techniques may provide opportunities for simple and rapid screening for the most effective additives. HTS techniques have become a valuable tool for speeding up process development at various stages of processing biopharmaceuticals [43,44]. They have been used effectively for rapid identification and selection of conditions for precipitation [45] and separation [46] of monoclonal antibodies from host cell proteins; for determining protein solubility [41,43] and rapid assessment of the dependence of colloidal stability on complex parameter interactions [41]; and for development of production processes [47] and quantification [48] of polysaccharide vaccine candidates. Mohr *et al*. [40] demonstrated a miniaturized HTS methodology for VLP formulation by integrating dynamic light scattering (DLS) and asymmetrical flow field-flow fractionation. DLS measurement with high-throughput capacity provides *in situ* analysis within short time periods using small amounts of protein. Although the DLS technique is a low resolution method with limitations and high sensitivity to the presence of large particles [40], it can be effectively used for HTS of protein processing conditions, particularly in cases when the size difference between different species, such as between capsomeres and soluble aggregates, is high.

The present study describes the development of a rapid, simple and effective HTS method to identify optimal processing conditions for stabilization of modular capsomeres. The method, based on DLS analysis, was used for rapid screening of subsets of buffer additives that could enhance the

stability of modular capsomeres (RvVP1), presenting an antigenic module derived from the VP8 subunit domain of the rotavirus spike protein. The potential of the superior additives based on DLS analysis in stabilizing RvVP1 was further confirmed with high-resolution SEC. The screening methodology developed in this study can be applied for tailoring the physicochemical environment of modular capsomeres and VLPs that incorporate antigenic modules having diverse physicochemical properties. The identification of protein-stabilizing buffer additives in this manner highlights the need to tailor the physicochemical environment specific to each module for processing and manufacture of stable modular capsomeres and VLPs in a fast and economical way. In this regard, the method can be used as a powerful tool for further advancing the platform and adding to the speed of manufacturing of quality modular capsomeres and VLPs for low-cost vaccine delivery at global scale.

3.2. Materials and methods

3.2.1. Chemicals

The following details the reagents used as additives and their source. All additives were of analytical grade: L-arginine (L-Arg) (MP Biomedicals, LLC Solon, Ohio, USA); L-glutamic acid (L-Glu), α-cyclodextrin, β-cyclodextrin, ϒ-cyclodextrin, methyl-β-cyclodextrin, tween-80 (TW-80), triton X-100 (TX-100), and bovine serum albumin (BSA) (Sigma–Aldrich, MO, USA); biotechnology grade phosphate buffered saline (PBS), and isopropyl-β-D-thiogalactoside (IPTG) (Astral Scientific Pty. Ltd., Gymea NSW, Australia); Tween-20 (TW-20) (Ajax Finechem, VIC, Australia).

3.2.2. Plasmid construction

Vector pGEX-4T-1 (GE Healthcare Biosciences, Chalfont St. Giles, UK) with inserted murine polyomavirus VP1 sequence (pGEXVP1) was generously provided by Professor Robert Garcea (University of Colorado, CO, USA). Plasmid, pGEXVP1-S4, encoding the protein, VP1, was created previously by inserting *AfeI* restriction enzyme site at position 293 of VP1 [15]. Plasmid, pGEXVP1-S4-VP8aa1-10, encoding RvVP1 was prepared from pGEXVP1-S4; RvVP1 is the protein VP1 but containing the amino acid sequence (EMASLIYRQLLESEMASLIYRQLLESEM ASLIYRQLLES) at position 293. The amino acid insert, VP8aa1-10, corresponds to tandem copies of residues 1-10 (MASLIYRQLL) of the VP8 subunit domain of the human rotavirus spike protein, with spacer amino acids. DNA sequence of the insert was prepared by PCR-based gene synthesis from a set of oligos (5´gagatggcgagcctcatct 3´, 5´cgctttcgaggagttggcgatagatgaggctcgccatctc3´, 5´gcc aactcctcgaaagcgaaatggcctctctgatctaccg3´, 5´atttcagactccagcagctggcggtagatcagagaggcca3´, 5´cagctg

ctggagtctgaaatggcgtccctgatttaccgtc3´and 5´ggattcgagcagttgacggtaaatcagggacgc3´), generated from DNAWorks (http: //helixweb.nih.gov/dnaworks/). Correct insertion of the desired gene sequence was verified by DNA sequencing at the Australian Genome Research Facility (Brisbane, Australia).

3.2.3. Protein expression and purification

pGEXVP1-S4 and pGEXVP1-S4-VP8$_{aa1-10}$ were transformed into separate *E. coli* Rosetta^{TM} (DE3) pLysS chemically competent cells (Novagen, San Diego, CA, USA). Bacterial expression of glutathione-S-transferase (GST) tagged proteins, GST-VP1 and GST-RvVP1, was as previously described [7,21] except cell cultures were induced with 0.1 mM IPTG at 20^{o}C for expression of GST-RvVP1. GST-tagged proteins were captured by GST affinity chromatography (GSTrapTM HP 5 ml column) (GE Healthcare) as described previously for purification of GST-VP1 [7,21,49]. All protein concentrations were determined using the Coomassie (Bradford) Protein Assay Kit (Pierce, IL, USA) microplate assay protocol. BSA was used as the standard and PBS was used to dilute the stock solution to achieve the standard concentrations in the assay.

3.2.4. Enzyme-mediated release of the GST tag

Thrombin-mediated cleavage to release the GST tag and purification of VP1 capsomeres was as previously reported [18,49]. For this process, purified GST-RvVP1 underwent thrombin proteolytic cleavage in a buffer containing different additives as summarized in Table 1. Briefly, concentrated stock solutions of additives listed in Table 1 were made in a buffer containing 40 mM tris-base, 1 mM ethylenediaminetetra-acetic acid (EDTA) disodium and 5% (v/v) glycerol. The additives were dissolved completely and filtered through 0.22 µm filter membrane [50]. The buffer was adjusted to pH 8.0 using aqueous solution of hydrochloric acid (32% (v/v)) and dithiothreitol (DTT) at a final concentration of 5 mM was added prior to use. Twenty-five microliters of freeze-thawed GST-RvVP1 aliquot at a concentration of 3.5 mg mL^{-1} in L buffer [200 mM NaCl, 40 mM tris (pH 8.0), 1 mM EDTA disodium, 5% (v/v) glycerol and 5 mM DTT], twenty-five microliters of buffer containing each additive (Table 1) and one microliter of thrombin (40 U/mL) (GE Healthcare) were mixed in 1.5 mL polypropylene Eppendorf tubes (Sarstedt, Australia). The concentration of each of the additives was adjusted to their target final concentration mentioned in Table 1, prior to mixing with the protein samples. The reaction mixtures were vortexed for 2 s, spun down using Wealtec E-centrifuge (Pathtech Pty Ltd, VIC, Australia) and then incubated at room temperature for 2 h. The reaction mixtures were then centrifuged at 22000g, 4°C, for 5.0 min, and the soluble fractions were analysed by SDS-PAGE and DLS measurements.

Table 3-1. List of buffer additives and their final concentrations for stabilization of modular capsomeres against aggregation

	Additives 1-27		Additives 28-54
1	200 mM NaCl	28	400 mM NaCl, 100 mM Trehalose
2	200 mM NaCl, 50 mM L-Arg	29	600 mM NaCl, 50 mM Trehalose
3	200 mM NaCl, 100 mM L-Arg	30	600 mM NaCl, 100 mM Trehalose
4	200 mM NaCl, 150 mM L-Arg	31	200 mM NaCl, 50 mM L-Arg, 50 mM L-Glu
5	200 mM NaCl, 200 mM L-Arg	32	200 mM NaCl, 10 mM α-cyclodextrin
6	200 mM NaCl, 250 mM L-Arg	33	200 mM NaCl, 10 mM β-cyclodextrin
7	150 mM NaCl	34	200 mM NaCl, 10 mM γ-cyclodextrin
8	150 mM NaCl, 50 mM L-Arg	35	200 mM NaCl, 10 mM Methyl-β-cyclodextrin
9	150 mM NaCl, 100 mM L-Arg	36	200 mM NaCl, 0.1% (v/v) TX-100
10	150 mM NaCl, 150 mM L-Arg	37	200 mM NaCl, 0.05% (v/v) TX-100
11	150 mM NaCl, 200 mM L-Arg	38	200 mM NaCl, 0.013% (v/v) TX-100
12	150 mM NaCl, 250 mM L-Arg	39	200 mM NaCl, 0.1% (v/v) TX-100, 25 mM L-Arg
13	100 mM NaCl	40	200 mM NaCl, 0.05% (v/v) TX-100, 25 mM L-Arg
14	100 mM NaCl, 50 mM L-Arg	41	200 mM NaCl, 0.013% (v/v) TX-100, 25 mM L-Arg
15	100 mM NaCl, 100 mM L-Arg	42	200 mM NaCl, 0.05% (v/v) TW-80
16	100 mM NaCl, 150 mM L-Arg	43	200 mM NaCl, 0.005% (v/v) TW-80
17	100 mM NaCl, 200 mM L-Arg	44	200 mM NaCl, 0.0013% (v/v) TW-80
18	100 mM NaCl, 250 mM L-Arg	45	200 mM NaCl, 0.05% (v/v) TW-80, 25 mM L-Arg
19	400 mM NaCl	46	200 mM NaCl, 0.005% (v/v) TW-80, 25 mM L-Arg
20	400 mM NaCl, 50 mM L-Arg	47	200 mM NaCl, 0.0013% (v/v) TW-80, 25 mM L-Arg
21	600 mM NaCl	48	200 mM NaCl, 0.05% (v/v) TW-20
22	600 mM NaCl, 50 mM L-Arg	49	200 mM NaCl, 0.023 % (v/v) TW-20
23	100 mM NaCl, 50 mM Trehalose	50	200 mM NaCl, 0.006% (v/v) TW-20
24	100 mM NaCl, 100 mM Trehalose	51	200 mM NaCl, 0.05% (v/v) TW-20, 25 mM L-Arg
25	200 mM NaCl, 50 mM Trehalose	52	200 mM NaCl, 0.023% (v/v) TW-20, 25 mM L-Arg
26	200 mM NaCl, 100 mM Trehalose	53	200 mM NaCl, 0.006% (v/v) TW-20, 25 mM L-Arg
27	400 mM NaCl, 50 mM Trehalose	54	200 mM NaCl, 25 mM L-Arg

3.2.5. Analysis of the cleavage products by SDS-PAGE

SDS-PAGE was performed using 10% SDS-PAGE gel as reported previously [11]. Gels were destained using 45% (v/v) methanol and 10% (v/v) acetic acid solution.

3.2.6. Dynamic light scattering (DLS) measurements

Soluble VP1 capsomere aggregates, VP1 capsomeres and GST were separated and purified by SEC as previously reported [18,49]. The fractions corresponding to the soluble VP1 aggregate peak were

pooled together and concentrated by ultracentrifugation at 5000g, 4°C for 5 min using Amicon® ultra-0.5 mL 10K membrane (Millipore Ireland Ltd, Tullagreen, Co. Cork, Ireland). The concentration of the protein was determined by Bradford microplate assay (Pierce, IL, USA). Similarly, the protein concentrations were determined for VP1 capsomere and GST peak fractions. The concentration of each sample was adjusted to 450 µg mL^{-1} by dilution with L buffer. Fifty microliters of solution mixtures were prepared by mixing solutions of pure VP1 capsomere aggregates (450 µg mL^{-1}), VP1 capsomeres (450 µg mL^{-1}) and GST (450 µg mL^{-1}) in 1.5 mL polypropylene microtubes (Sarstedt, Australia). All solution mixtures contained 50% (v/v) GST solution, and from 0% - 50% (v/v) VP1 capsomere and aggregate solutions. Pure solutions of VP1 capsomere aggregates (450 µg mL^{-1}), VP1 capsomeres (450 µg mL^{-1}) and GST (450 µg mL^{-1}), and their mixtures were subjected to analysis by DLS for particle size and size distribution in order to validate DLS analysis method using a 384-well microplate. Briefly, 20 µL of the mixtures were transferred in duplicates in to a 384-well Corning® polystyrene plate (Corning, NY, USA), and centrifuged to remove trapped air bubbles at 1000g, 4°C, for 4 min. Analyses were performed by using a Dynapro® plate reader (Wyatt Technology Corporation, CA, USA) equipped with an 830 nm laser and a temperature control module. The Dynamics® software (Version 7.0.3.12, Wyatt Technology Corporation) was used for scheduled data acquisition and analysis. Ten 5 s measurements were taken for each well, resulting in 20 measurements for each mixture at 25°C. Data analysis was performed using the Dynals algorithm bundled with the Dynamics® software. The DLS method that was validated in this manner was applied to HTS of buffer additives for their potential to stabilize RvVP1 capsomeres following thrombin-mediated release of the GST tag in the presence of additives summarized in Table 1.

3.2.7. Analysis by size exclusion chromatography

Separation and purification of VP1 capsomeres by SEC was performed as previously described [18,21]. Purification of RvVP1 capsomeres followed similar approaches. Briefly, GST-RvVP1 was diluted to 1.75 mg mL^{-1} with L buffer or L buffer containing TX-100, TW-80, or TW-20, alone or in combination with 25 mM L-Arg at final concentrations as described in Table 1 and subjected to thrombin-mediated release of GST tag at room temperature for 2 h. The cleavage products were centrifuged (22,000g, 4°C, 5 min) and RvVP1 capsomeres were recovered from 500 µL of the supernatant with a Superdex 200 30/100 GL column (GE Healthcare Biosciences) operated with an AKTAexplorer™10 (GE Healthcare Biosciences) liquid chromatography system. The column was pre-equilibrated with L buffer or L buffer containing each of the additives at a flow rate of 0.5 mL min^{-1}. Proteins were detected and visualized with SDS-PAGE from elution fractions corresponding to the aggregate and capsomere peaks of SEC chromatograms.

3.3. Results and Discussion

3.3.1. Expression and purification of GST-VP1 and GST-RvVP1

Expression of GST fusion proteins by *E. coli* Rosetta (DE3) pLysS cells transformed with the relevant expression vectors was confirmed by SDS-PAGE (Fig. 3-1A). No basal expression of the target recombinant GST fusion proteins was observed from the cultures harvested before induction with IPTG. Target proteins were found in the soluble fractions of the cell lysates of induced cultures. Purification by GST affinity chromatography resulted in a good recovery of target GST fusion protein (Fig. 3-1B).

GST-VP1 and GST-RvVP1 were subjected to enzymatic cleavage with thrombin as reported previously [7,21]. SDS-PAGE analysis of the soluble fraction following cleavage (Fig. 3-2A) showed that the enzyme was able to release the tag completely. Cleavage product mixtures were subjected to SEC for separation and purification of capsomeres, soluble capsomere aggregates and GST for both VP1 and RvVP1 (Fig. 3-2B). The lanes in Fig. 3-2A demonstrate that the majority of soluble VP1 protein (Lane 3) remains as capsomeres (Lane 4); whereas for RvVP1 the majority of the soluble protein (Lane 6) is lost in the aggregate peak (Lane 7) and barely any remains as capsomeres (Lane 8). This observation demonstrates that RvVP1 capsomeres were not stable when the stability buffer optimized for VP1 capsomere [21] was used for downstream processing of RvVP1. The specific causes for poor stability of RvVP1 capsomeres are unknown. It is speculated that instability of the capsomeres may be driven by hydrophobic interactions. Thus, modularization of capsomeres can necessitate re-optimisation of the buffer conditions to maintain the colloidal stability of the new protein that results from modularisation.

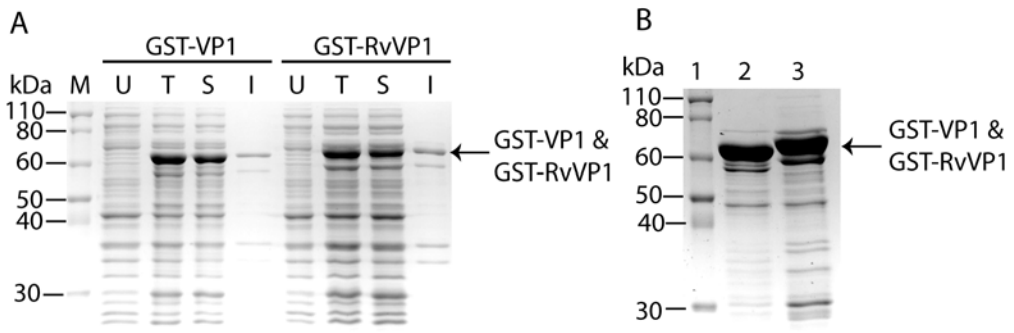

Figure 3-1. Expression profile of GST-VP1 and GST-RvVP1 in E. coli Rosetta (DE3) pLysS cells. (A) The target proteins were visualized by SDS-PAGE from un-induced (U) cultures, total cell lysate (T), soluble fraction (S) and insoluble fraction (I) of induced cultures. Novex® Sharp Pre-stained Protein marker (M) was used as ladder. (B) The target GST fusion proteins (Lane 1: Protein marker; Lane 2: GST-VP1 and; Lane 3: GST-RvVP1) were purified from the soluble fractions of the cell lysates by GST affinity chromatography and visualized by SDS-PAGE.

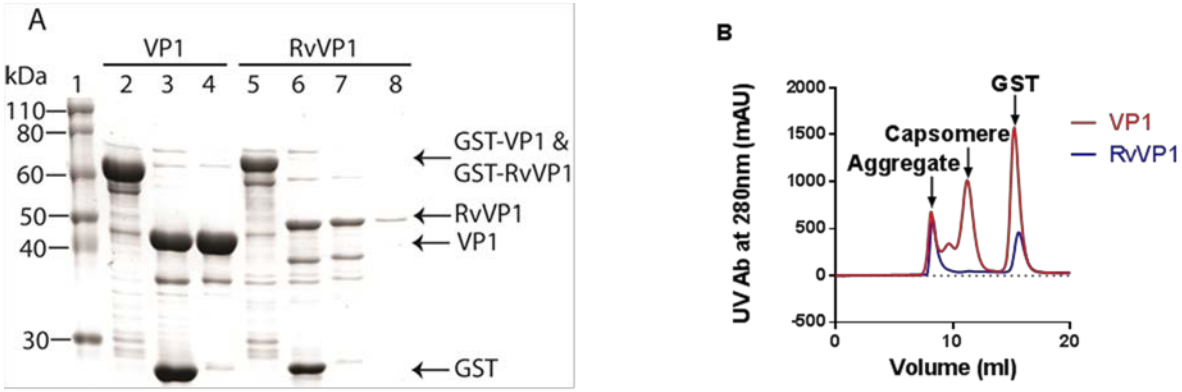

Figure 3-2. Relative solubility of VP1 and RvVP1 capsomere in L-buffer optimised for VP1. (A) SDS-PAGE analysis of the target proteins (Lane 1: Protein marker; Lane 2: GST-VP1; Lane 3: Total soluble protein after cleavage of GST-VP1; Lane 4: VP1 capsomere peak fraction; Lane 5: GST-RvVP1; Lane 6: Total soluble protein after cleavage of GST-RvVP1; Lane 7: RvVP1 aggregate peak fraction and; Lane 8: RvVP1 capsomere peak fraction) during downstream processing. (B) Size exclusion chromatogram of VP1 and RvVP1 capsomeres following thrombin mediated cleavage of the GST tag in L-buffer.

3.3.2. DLS analysis for HTS of buffer additives for enhanced stability of modular capsomeres

3.3.2.1. Validation of the DLS method

DLS is a non-invasive diagnostic tool for particle sizing and determination of particle size distribution in solutions or colloidal dispersions [50]. From DLS measurements, diffusion coefficient can be obtained from analysis of time-dependent fluctuations of scattered light intensity arising from particles undergoing random Brownian motion [40,51]. The hydrodynamic radius or the Z-average radius (or cumulant mean) of particles can be calculated from the molecular diffusion

coefficient using the Stokes-Einstein equation [51]. However, due to DLS particle sizing´s higher sensitivity to the presence of large particles, such as soluble aggregates [40], accurate interpretation of the DLS data is very challenging. The presence of trace amounts of large particles may mask contributions of small particles on the over-all hydrodynamic property of solutions. Thus validation of DLS analysis using well characterized materials can minimize errors and avoid a false negative or false-positive conclusion.

Solutions of pure VP1 capsomeres, VP1 capsomere aggregates and GST, purified by SEC and quantified by Bradford microplate assay, and their solution mixtures [section 2.5] were used to validate the DLS measurement, using a 384-well plate. Mixtures of VP1 capsomeres, VP1 aggregates and GST solutions were used to simulate the thrombin cleavage products of GST-RvVP1 in the presence of various additives (Table 1). Fig. 3-3A shows the change in the Z-average radius of particles dependent on the ratio of capsomere and aggregate particles in solution. As % (v/v) of capsomere solution increased from 0 to 50%, the Z-average radius decreased. As expected, the Z-average radius was highest for the solution containing pure capsomere aggregates and the lowest for the solution of pure GST. The Z-average radius is a preferred parameter for particle size analysis by DLS since its calculation is mathematically stable and the Z-average result is less sensitive to noise [52,53].

Fig. 3B shows the intensity autocorrelation curve for pure VP1 capsomere, VP1 capsomere aggregate and GST solutions, and solution mixtures. The autocorrelation curves at 100%, 50% and 45% (v/v) of VP1 capsomeres show a smooth and continuous exponential decay, indicative of low sample polydispersity. The decay time for particles in the mixture extended when the % (v/v) of the capsomere solution decreased from 50% to 40% or the % (v/v) of capsomere aggregate solution increased from 0 to 10%. The extended decay time might be due to the greater sample polydispersity. Moreover, diffusion of particles might be slowed down due to inter-particle interactions of small and large particles at some critical number of particles, creating an optimum surface area for maximum interaction to occur, affecting the hydrodynamic properties of the particles [54]. The DLS analysis method, which was validated using artificial mixtures of purified VP1 capsomeres, VP1 capsomere aggregates and GST, was applied to HTS of buffer additives for their potential to enhance the stability of RvVP1 capsomeres.

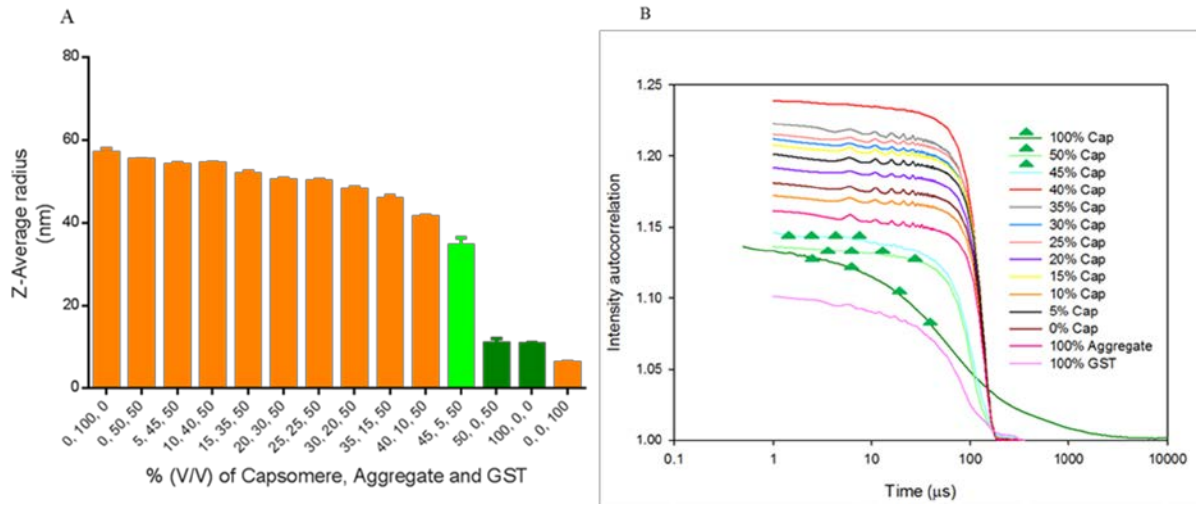

Figure 3-3. Validation of the DLS method using solution mixtures of pure VP1capsomeres, VP1 capsomere aggregates and the GST. (A) Plot of the Z-average radius of particles in the mixture against % (v/v) of VP1 capsomeres, aggregates and GST respectively in solution mixtures. The Z-Average radius expressed as mean of two values. The coloured bars, dark green (■), light green (■) and orange (■), present good, slightly good and bad conditions respectively. (B) DLS showing the intensity autocorrelation of the mixtures with time. The good conditions are indicated by▲.

3.3.2.2. HTS of buffer additives for enhanced stability of modular capsomeres

Buffer additives (Table 1) including NaCl at different concentrations, L-Arg, equimolar mixture of L-Arg and L-Glu, trehalose, cyclodextrins, the non-ionic detergents, TX-100, TW-80 and TW-20, and combinations of L-Arg and non-ionic detergents were examined for their potential to enhance stability of RvVP1 capsomeres. The concentration of the additives was fixed based on their use in the literature as stabilizing agents at various stages of protein processing, formulation or upon storage [30,33,40,55] and in consideration of their compatibility with the activity of thrombin and the DLS measurement. The additives were added to the protein solution before release of the GST tag using thrombin. Successful cleavage of the tag was assessed by SDS-PAGE. It was observed that L-Arg at a concentration of 100, 150, 200 and 250 mM, and equimolar mixtures of L-Arg and L-Glu at 50 mM, decreased the enzymatic cleavage activity of thrombin (data not shown). In a previous study it was speculated that L-Arg and L-Glu may act as strong competitive inhibitors of proteases or that binding of L-Arg and L-Glu to the surface of the protein may mask the protease recognition site [30]. All other additives did not interfere with the action of the enzyme for GST tag release under the conditions tested.

HTS technologies are based on fast analysis and low sample volume [42,43] meaning that a large number of conditions can be quickly tested using a relatively small amount of materials [40,42]. Here we have developed a DLS-based method that allows quick screening of relatively low sample

volume (20 µL) to monitor the stability of RvVP1 capsomeres in the presence of additives listed in Table 1. According to the DLS method validation (Fig. 3-3) reduction in the Z-average radii of particles (less than 40 nm) indicates the ability of theses additives to enhance the stability of modular capsomeres (size ~ 10 nm) and inhibit the formation of large aggregates (size > 40 nm). The results of the DLS analysis of the additive screen are summarized in Fig. 3-4, which depicts the Z-average radius of particles in cleavage product solutions plotted against processing conditions categorized into 17 subgroups based on the type or concentration of additives. The Z-average radii of particles were greater than 40 nm for most of the conditions tested in this study; however, the evaluation of the 54 conditions (Table 1) demonstrated better performance of eight conditions (Fig. 3-4). These conditions contained non-ionic detergents, 0.1% (v/v) TX-100, 0.05% (v/v) TX-100, TW-80 and TW-20 as additives either alone or in combination with 25 mM L-Arg. It is common for buffers to be augmented with detergents to increase solubility and to maintain stability during isolation and purification of hydrophobic proteins [56,57]. Among the different types of detergents, the use of non-ionic detergents to stabilize proteins, suppress aggregation and assist protein refolding has been well documented in the literature [31,55,57-60]. Their amphiphilic nature makes them good competitors with proteins for surfaces and interfaces and thus prevent adsorption induced denaturation and aggregation of proteins [31,60]. In addition, non-ionic surfactants may directly interact with hydrophobic regions in protein molecules and reduce their tendency to aggregate [61,62]. They can also prevent aggregation by serving as chaperones assisting protein folding and refolding [62].

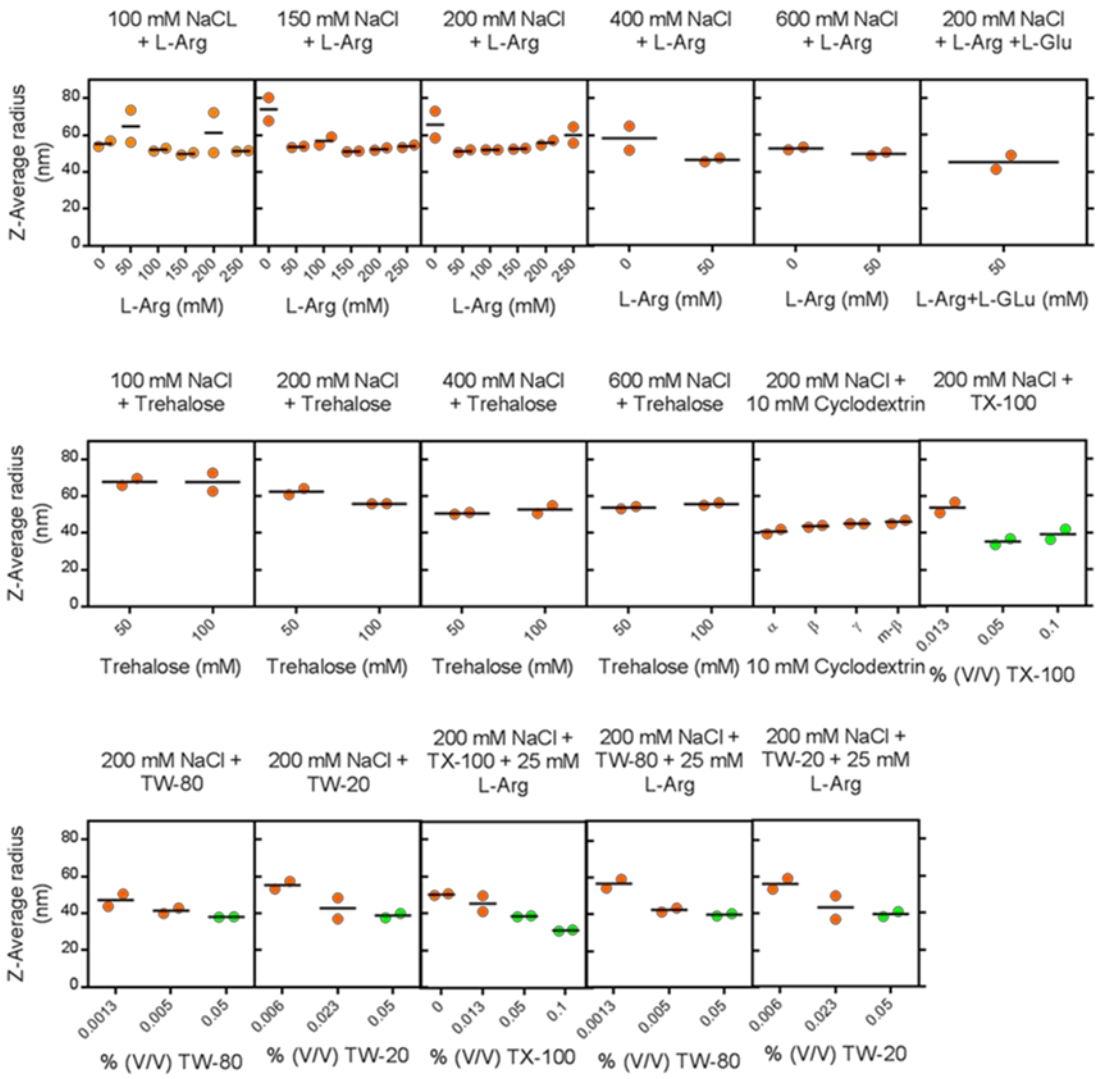

Figure 3-4. DLS-based HTS of buffer additives for enhanced stability of modular RvVP1 capsomeres. The Z-average radius of particles in solutions of the cleavage product mixtures plotted against the cleavage buffer additives. The Z-average radius expressed as mean of two values. The values of Z-average radii of particles in the presence of promising additives indicated by green ball (●) and the orange ball (●) represents the values in the presence of less performing additives.

The potential of TX-100, TW-80 and TW-20 either alone or in combination with L-Arg to increase the stability of RvVP1 capsomeres was further confirmed with high-resolution SEC. Following thrombin-mediated cleavage to release the GST tag in the presence of TX-100 (0.1% and 0.05% (v/v) alone or in combination with 25 mM L-Arg, SEC showed that the detergent was able to increase the stability of modular capsomeres (Fig. 3-5) compared with downstream processing without the additives (Fig. 3-2B). Similarly, TW-80 and TW-20 (0.05% (v/v)) alone or in combination with 25 mM L-Arg could also increase stability of modular capsomeres (Fig. 3-5). L-Arg alone was not able to enhance stability of modular capsomeres (data not shown) and there were no clear differences between the mAU UV absorbance values of the peaks corresponding to RvVP1

capsomere fractions when detergents were used alone and in combination with L-Arg as stabilizing additives (Fig. 3-5). These results indicate that enhanced stability of modular capsomeres was mainly due to the non-ionic detergents, and the use of L-Arg in combination with detergents did not result in additive or synergistic effect to enhance the stability of RvVP1 capsomeres. The presence of L-Arg can greatly influence the stability of a protein in solution. L-Arg is one of the most widely used aggregation-suppressing additives during protein refolding and purification [63-67]. However, it may not work well for all proteins and its effect depends on protein-additive or intra-additive interactions determined by the concentration of the additive [68].

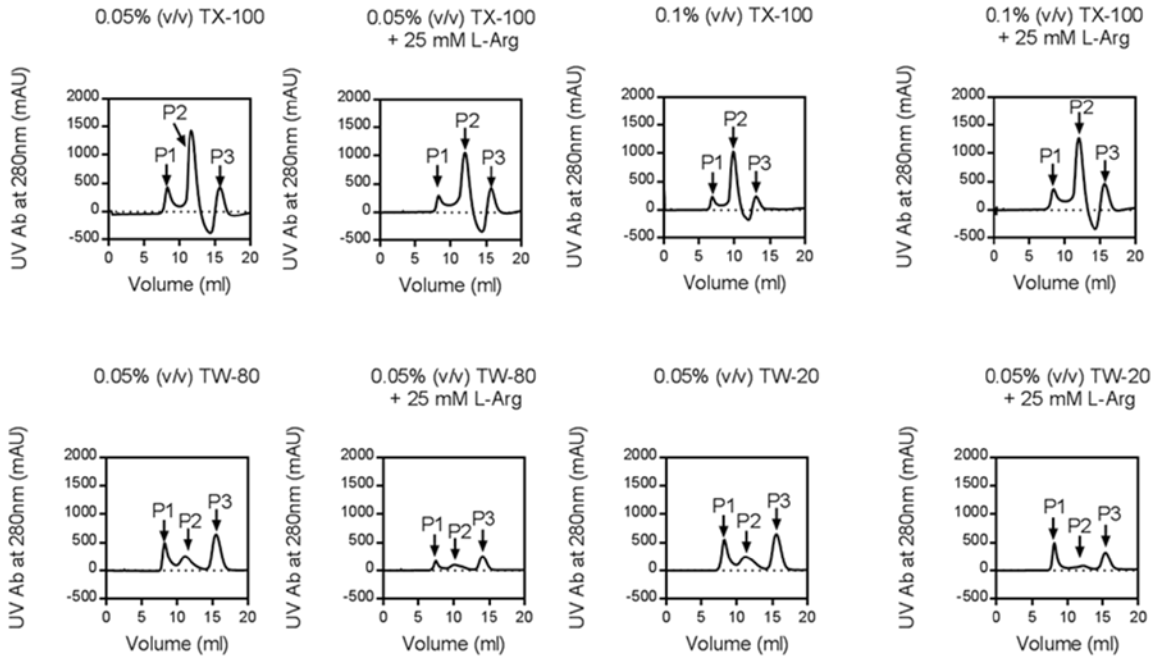

Figure 3-5. Size exclusion chromatogram of RvVP1 capsomeres following thrombin mediated cleavage of the GST tag in L-buffer with TX-100, Tween and L-Arg additives. P1, P2 and P3 represent the aggregate, capsomere and GST peaks respectively.

To ensure the improved capsomere stability observed in SEC traces was not an artefact of the buffer itself, the RvVP1 protein was visualized with SDS-PAGE (Fig. 3-6) from the cleavage product mixtures and from purified SEC fractions. As shown in Fig. 3-6 A, the use of non-ionic detergent (TX-100, TW-80 or TW-20) as buffer additive during downstream processing could increase the stability of RvVP1 capsomeres compared with processing in buffer conditions optimized for VP1 capsomeres (Fig. 3-2 A, lanes 7 and 8). Concentration (C) of RvVP1 protein from capsomere peak fractions (Fig. 3-5) was determined using Bradford microplate assay. The result showed that there was no clear difference in protein concentration when modular capsomeres were processed using

TX-100 [C = 0.206 mg/mL), TW-80 [C = 0.184 mg/mL] or TW-20 [C = 0.192 mg/mL] as stabilizing additive. Despite its application in enhancing stability of proteins and their elution from chromatographic resins during purification [65,69], use of L-Arg in combination with non-ionic detergents did not increase the yield of purified modular capsomeres as the concentration of the protein was almost the same as proteins purified using non-ionic detergents as the sole additives. Generally, the high-resolution SEC and subsequent analyses were in agreement with the results obtained from the HTS approach using DLS; improved capsomere yield means a higher population of particles in capsomere size range, which is reflected in the DLS Z-average size. Thus, there is agreement between the results obtained from DLS analysis and SEC, confirming the validity of this approach. The HTS method demonstrated in this study is simple, rapid and effective approach for identifying suitable buffer additives to increase stability of modular capsomeres. While the method demonstrated here was limited in the use of lab scale facilities, the process itself could be utilized in larger scale, higher throughput facilities, thus justifying it as a high-throughput screen. Here it could identify eight potential processing conditions to enhance stability of RvVP1 capsomeres out of 54 conditions in less than two hours. Moreover, such a method saves substantial amount of protein sample and buffers compared to an iterative trial and error approach of process optimization using analytical tools having low-throughput capacity.

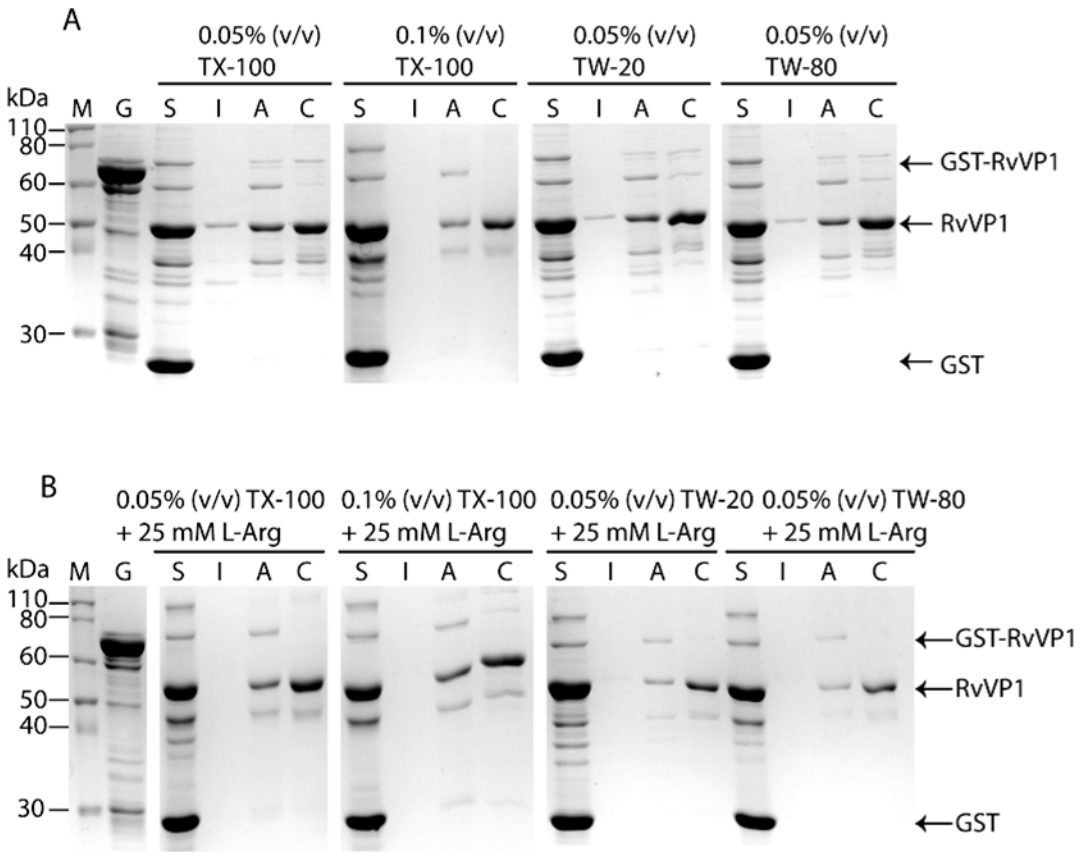

Figure 3-6. SDS-PAGE analysis of the target proteins (marker protein (M), GST-RvVP1 (G), total soluble protein after cleavage (S), total insoluble protein after cleavage (I), aggregate peak fraction (A) and capsomere peak fraction (C)) throughout the downstream processing steps using: (A) non-ionic detergents as buffer additives; and (B) combination of L-Arg and non-ionic detergents as buffer additives.

3.4. Conclusion

Our aim was to develop a HTS method for rapid identification of buffer additives to increase stability of modular capsomeres during downstream processing. The method, based on a high-throughput DLS analysis, has identified small organic molecules that could increase stability of RvVP1 capsomere, which was used as a model protein in this study. The method is simple and effective, requiring little analysis time and low protein consumption. Using this strategy, 54 processing conditions in the presence of various additives were screened for their potential to enhance stability of RvVP1 capsomeres. Eight conditions were successfully identified as effective stabilisers, from which the additives, 0.1% (v/v) TX-100, 0.05% (v/v) TX-100, TW-80 and TW-20 were able to increase stability of RvVP1 modular capsomeres. The potential of these subsets of additives for enhancing stability of modular capsomeres during purification was further confirmed with a high-resolution SEC. Non-ionic detergents are very mild chemical compounds that do not denature proteins and they increase stability of proteins by inhibiting hydrophobic interactions between proteins or by coating interfaces to modulate adsorption loss and aggregation. The results

in this study demonstrate that tailoring the nature of the physicochemical environment surrounding the proteins using small organic molecules could stabilize RvVP1 modular capsomeres. The developed screening method provides a powerful approach for rapid tailoring of processing conditions specific to the antigenic modules on modular capsomeres. It can also eliminate time-consuming and laborious trial and error approaches to optimize processing conditions for other proteins at all stages of purification.

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References

1. Castro-Acosta RM, Revilla AL, Ramirez OT, Palomares LA: **Separation and quantification of double- and triple-layered rotavirus-like particles by capilary zone electrophoresis**. *Electrophoresis* 2010, **31**:1376-1381.
2. Vicente T, Roldão A, Peixoto C, Carrondo MJT, Alves PM: **Large-scale production and purification of VLP-based vaccines**. *Journal of Invertebrate Pathology* 2011, **107**:S42-S48.
3. Lua LHL, Connors NK, Sainsbury F, Chuan YP, Wibowo N, Middelberg APJ: **Bioengineering virus like-particles as vaccines**. *Biotechnollogy and Bioengineering* 2014, **111**:425-440.
4. Zeltins A: **Constructions and characterizations of virus-like particles: a review**. *Molecular Biotechnology* 2012, **53**:92-107.
5. Benavides J, Mena JA, Cisneros-Ruiz M, Ramirez OT, Palomares LA, Rito-Palomares M: **Rotavirus-like particles primary recovery from insect cells in aqueous two-phase systems**. *Journal of Chromatography B* 2006, **842**:48-57.
6. Peralta A, Molinari P, Taboga O: **Chimeric recombinant rotavirus-like particles as a vehicle for the display of heterologous epitopes**. *Virology Journal* 2009, **6**:192-200.
7. Liew MWO, Rajendran A, Middelberg APJ: **Microbial production of virus-like particle vaccine protein at gram-per-litre levels**. *Journal of Biotechnology* 2010, **150**:224-231.
8. Kang SM, Kim MC, Compans RW: **Virus-like particles as universal influenza vaccines**. *Expert Reviews Vaccines* 2012, **11**:995-1007.
9. Crisci E, Barcena J, Montoya M: **Virus-like particles: the new frontier of vaccines for animal viral infections**. *Veterinary Immunology and Immunopathology* 2012, **148**:211-225.
10. Kushnir N, Streatfield SJ, Yusibov V: **Virus-like particles as a highly efficient vaccine platform: Diversity of targets and production systems and advances in clinical development**. *Vaccine* 2012, **31**:58-83.
11. Connors NK, Wu Y, Lua LHL, Middelberg APJ: **Improved fusion tag cleavage strategies in the downstreamprocessing of self-assembling virus-like particle vaccines**. *Food and Bioproducts Processing* 2014, **92**:143-151.
12. Tan M, Jiang X: **Subviral particle as vaccine and vaccine platform**. *Current Opinion Virology* 2014, **6**:24-33.
13. Zhao Q, Li S, Yu H, Xia N, Modis Y: **Virus-like particle-based human vaccines: quality assessment based on structural and functional properties**. *Trends Biotechnology* 2013, **31**:654-663.

14. Rodriguez-Limas WA, Tyo KEJ, Nielsen J, Ramirez OT, Palomares LA: **Molecular and process design for rotavirus-like particle production in *Saccharomyces cerevisiae***. *Microbial Cell Factories* 2011, **10**:DOI: 10.1186.

15. Middelberg APJ, Rivera-Hernandez T, Wibowo N, Lua LHL, Fan Y, Magor G, Chang C, Chuan YP, Good MF, Batzloff MR: **A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines**. *Vaccine* 2011, **29**:7154-7162.

16. Laimbacher AS, Esteban LE, Castello AA, Cerfoglio JCA, Argüelles MH, Glikmann G, Antuono AD, Mattion N, Berois M, Arbiza J, et al.: **HSV-1 amplicon vectors launch the production of heterologous rotavirus-like particles and induce rotavirus-specific immune responses in mice**. *Molecular Therapy* 2012, **20**:1810-1820.

17. Schädlich L, Senger T, Kirschning CJ, Müller M, Gissmann L: **Refining HPV 16 L1 purification from *Escherichia coli*: Reducing endotoxin contaminations and their impact on immunogenicity**. *Vaccine* 2009, **27**:1511-1522.

18. Wibowo N, Chuan YP, Lua LHL, Middelberg APJ: **Modular engineering of a microbially-produced viral capsomere vaccine for influenza**. *Chemical Engineering Science* 2012, **103**:12-20.

19. Dell K, Koesters R, Linnebacher M, Klein C, Gissmann L: **Intranasal immunization with human papillomavirus type 16 capsomeres in the presence of non-toxic cholera toxin-based adjuvants elicits increased vaginal immunoglobulin levels**. *Vaccine* 2006, **24**:2238-2247.

20. Gissmann L: **HPV vaccines: preclinical development**. *Archives of Medical Research* 2009, **40**:466-470.

21. Chuan YP, Lua LHL, Middelberg APJ: **High-level expression of soluble viral structural protein in *Escherichia coli***. *Journal of Biotechnology* 2008, **134**:64-71.

22. Ribeiro-Müller L, Müller M: **Prophylactic papillomavirus vaccines**. *Clinical Dermatology* 2014, **32**:235-247.

23. Chuan YP, Wibowo N, Lua LHL, Middelberg APJ: **The economics of virus-like particles and capsomere vaccines**. *Biochemical Engineering Journal* 2014, **90**:255-263.

24. Liew MWO, Chuan YP, Middelberg APJ: **High-yield and scalable cell-free assembly of virus-like particles by dilution**. *Biochemical Engineering Journal* 2012, **67**:88-96.

25. Noad R, Roy P: **Virus-like particles as immunogens**. *Trends Microbiology* 2003, **11**:438-444.

26. Liew MWO, Chuan YP, Middelberg APJ: **Reactive diafiltration for assembly and formulation of virus-like particles**. *Biochemical Engineering Journal* 2012, **68**:120-128.

27. Rivera-Hernandez T, Hartas J, Wu Y, Chuan YP, Lua LHL, Good M, Batzloff MR, Middelberg APJ: **Self-adjuvanting modular virus-like particles for mucosal vaccination against group A streptococcus (GAS)**. *Vaccine* 2013, **31**:1950-1955.

28. Wibowo N, Hughes FK, Fairmaid EJ, Lua LHL, Brown LE, Middelberg APJ: **Protective efficacy of a bacterially produced modular capsomere presenting M2e from influenza: extending the potential of broadly cross-protecting epitopes**. *Vaccine* 2014, **32**:3651-3655.

29. Ajmera A, Scherließ R: **Stabilisation of proteins via mixtures of amino acids during spray drying**. *International Journal of Pharmaceutics* 2014, **463**:98-107.

30. Golovanov AP, Hautbergue GM, Wilson SA, Lian LY: **A simple method for improving protein solubility and long-term stability**. *Journal of American Chemical Society* 2004, **126**:8933-8939.

31. Lee HJ, McAuley A, Schilke KF, McGuire J: **Molecular origins of surfactant-mediated stabilization of protein drugs**. *Advanced Drug Delivery Reviews* 2011, **63**:1160-1171.

32. Matsuoka T, Tomita S, Hamada H, Shiraki K: **Amidated amino acids are prominent additives for preventing heat-induced aggregation of lysozyme**. *Journal of Bioscience and Bioengineering* 2007, **103**:440-443.

33. Leibly DJ, Nguyen TN, Kao LT, Hewitt SN, Barrett LK, Van Voorhis WC: **Stabilizing additives added during cell lysis aid in the solubilization of recombinant proteins**. *PloS One* 2012, **7**:e52482.

34. Hamada H, Shiraki K: **L-Argininamide improves the refolding more effectively than L-arginine**. *Journal of Biotechnology* 2007, **130**:153-160.

35. Tsumoto K, Umetsu M, Kumagai I, Ejima D, Philo JS, Arakawa T: **Role of arginine in protein refolding, solubilization and purification**. *Biotechnology Progress* 2004, **20**:1301-1308.

36. Stenvall M, Steen J, Uhlen M, Hober S, Ottosson J: **High-throughput solubility assay for purified recombinant protein immunogens**. *Biochimica et Biophysica Acta* 2005, **1752**:6-10.

37. Vincentelli R, Canaan S, Campanacci V, Valencia C, Maurin D, Frassinetti F, Scappucini-Calvo, Bourne Y, Cambillau C, Bignon C: **High-throughput automated refolding screening of inclusion bodies**. *Protein Science* 2004, **13**:2782-2792.

38. Kumar N, Kishore N: **Protein stabilization and counteraction of denaturing effect of urea by glycine betaine**. *Biophysical Chemistry* 2014, **189**:16-24.

39. Kumar N, Kishore N: **Structure and effect of sarcosine on water and urea by using molecular dynamics simulations: implications in protein stabilization**. *Biophysical Chemistry* 2013, **171**:9-15.

40. Mohr J, Chuan YP, Lua LHL, Middelberg APJ: **Virus-like particle formulation optimization by miniaturized high-throughput screening**. *Methods* 2013, **60**:248-256.

41. Berg A, Schuetz M, Dismer F, Hubbuch J: **Automated measurement of apparent protein solubility to rapidly assess complex parameter interactions**. *Food and Bioproducts Processing* 2014, **92**:133-142.

42. Dasnoy S, Dezutter N, Lemoine D, Le Bras V, Preat V: **High-throughput screening of excipients intended to prevent antigen aggregation at air-liquid interface**. *Pharmaceutical Research* 2011, **28**:1591-1605.

43. Wiendahl M, Volker C, Husemann I, Krarupa J, Staby A, Scholl S, Hubbuch J: **A novel method to evaluate protein solubility using a high-throughput screening approach**. *Chemical Engineering Science* 2009, **64**:3778-3788.

44. Hanke AT, Ottens M: **Purifying biopharmaceuticals: knowledge-based chromatographic process development**. *Trends in Biotechnology* 2014, **32**:210-220.

45. Knevelman C, Davies J, Lee A, Titchener-Hooker NJ: **High-throughput screening techniques for rapid PEG-based precipitation of IgG_4 monoclonal antibody from clarified cell culture supernatant**. *Biotechnology Progress* 2009, **26**:697-705.

46. Oelmeier SA, Ladd-Effio C, Hubbuch J: **Alternative separation steps for monoclonal antibody purification: combination of centrifugal partitioning chromatography and precipitation**. *Journal of Chromatography A* 2013, **1319**:118-126.

47. Noyes A, Godavarti R, Titchener-Hooker N, Coffman J, Mukhopadhyay T: **Quantitative high-throughput analytics to support polysaccharide production process development**. *Vaccine* 2014, **32**:2819-2828.

48. Noyes A, Boesch A, Godavarti R, Titchener-Hooker N, Coffman J, Mukhopadhyay T: **High-throughput quantification of capsular polysaccharides for multivalent vaccines using precipitation with a cationic surfactant**. *Vaccine* 2013, **31**:5659-5665.

49. Lipin DL, Lua LHL, Middelberg APJ: **Quaternary size distribution of soluble aggregates of glutathione-S-transferase purified viral protein as determined by asymmetrical flow field flow fractionation and dynamic light scattering**. *Journal of Chromatography A* 2008, **1190**:204-214.

50. Li Z, Wang Y, Shen J, Liu W, Sun X: **The measurement system of nanoparticle size distribution from dynamic light scatering data**. *Optics and Lasers in Engineering* 2014, **56**:94-98.

51. Alatorre-Meda M, Taboada P, Sabin J, Krajewska B, Varela LM, Rodriguez JR: **DNA-chitosan complexation: a dynamic light scattering study**. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 2009, **339**:145-152.

52. Frisken BJ: **Revisiting the method of cumulants for the analysis of dynamic light scattering data**. *Applied optics* 2001, **40**:4087-4091.

53. Koppel DE: **Analysis of molecular polydispersity in intensity correlation spectroscopy: the method of cumulants** *Journal of Chemical Physics* 1972, **57**:4814.

54. Adamczyk Z, Sadlej K, Wajnryb E, Ekiel-Jezewska ML, Warszynski P: **Hydrodynamic radii and diffusion coefficients of particle aggregates derived from the bead model**. *Journal of Colloid and Interface Science* 2010, **347**:192-201.

55. Bondos SE, Bicknell A: **Detection and prevention of protein aggregation before, during and after purification**. *Analytical Biochemistry* 2003, **316**:223-231.

56. Kragh-Hansen U, Le Maire M, Møller JV: **The mechanism of detergent solubilization of liposomes and protein-containing membranes**. *Biophysical Journal* 1998, **75**:2932-2946.

57. Seddon AM, Curnow P, Booth PJ: **Membrane proteins, lipids and detergents: not just a soap opera**. *Biochimica et Biophysica Acta-Biomembranes* 2004, **1666**:105-117.

58. Wang W, Wang YJ, Wang DQ: **Dual effects of tween 80 on protein stability**. *International Journal of Pharmaceutics* 2008, **347**:31-38.

59. Chou D, Krishnamurthy R, Randolph T, Carpenter J, Manning M: **Effects of tween 20 and tween 80 on the stability of Albutropin during agitation**. *Journal of Pharmaceutical Sciences* 2005, **94**:1368-1381.

60. Joshi O, Chu L, McGuire J, Wang DQ: **Adsorption and function of recombinant Factor VIII at the air-water interface in the presence of tween 80**. *Journal of Pharmaceutical Sciences* 2009, **98**:3099-3107.

61. Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB: **Protein-excipient interactions: mechanisms and biophysical characterization applied to protein formulation development**. *Advanced Drug Delivery Reviews* 2011, **63**:1118-1159.

62. Ohtake S, Kita Y, Arakawa T: **Interactions of formulation excipients with proteins in solution and in the dried state**. *Advanced Drug Delivery Reviews* 2011, **63**:1053-1073.

63. Arakawa T, Tsumoto K: **The effects of arginine on refolding of aggregated proteins: not facilitate refolding, but suppress aggregation**. *Biochemical and Biophysical Research Communications* 2003, **304**:148-152.

64. Baynes BM, Wang DIC, Trout BL: **Role of arginine in the stabilization of proteins against aggregation**. *Biochemistry* 2005, **44**:4919-4925.

65. Ishibashi M, Tsumoto K, Tokunaga M, Ejima D, Kita Y, Arakawa T: **Is arginine a protein-denaturant?** *Protein Expression and Purification* 2005, **42**:1-6.

66. Ho JGS, Middelberg APJ: **Estimating the potential refolding yield of recombinant proteins expressed as inclusion bodies**. *Biotechnology and Bioengineering* 2004, **87**:584-592.

67. Ho JGS, Middelberg APJ, Ramage P, Kocher HP: **The likelihood of aggregation during protein renaturation can be assessed using the second viral coefficient** *Protein Science* 2003, **12**:708-716.

68. Shukla D, Schneider CP, Trout BL: **Molecular level insight into intra-solvent interaction effects on protein stability and aggregation**. *Advanced Drug Delivery Reviews* 2011, **63**:1074-1085.

69. Arakawa J, Uegaki M, Ishimizu T: **Effects of L-arginine on solubilization and purification of plant membrane proteins**. *Protein Expression and Purification* 2011, **80**:91-96.

Chapter 4

Design strategies to address the effect of hydrophobic epitope on stability and in vitro assembly of modular virus-like particle

The entire Chapter 4 consists of the journal article submitted as:

Alemu Tekewe, Natalie K. Connors, Anton P.J. Middelberg, Linda H.L. Lua. Design strategies to address the effect of hydrophobic epitope on stability and *in vitro* assembly of modular virus-like particle. Protein Science 2016, 25: 1507-1516.

The following modifications were made to the original submitted article:

- Page numbers of the original submitted article were changed into the numbers consistent with those on the remainder of the thesis page numbers.
- Figure numbers were changed into the numbers consistent with those on the remainder of figure in the thesis.
- Section and sub-section numbers were changed into the numbers consistent with those on the remainder of sections and sub-section numbers in all chapters of the thesis.
- The reference style of the original submitted article was changed into the style consistently used in all chapters of the thesis.

Design strategies to address the effect of hydrophobic epitope on stability and *in vitro* assembly of modular virus-like particle

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Abstract

Virus-like particles (VLPs) and capsomere subunits have shown promising potential as safe and effective vaccine candidates. They can serve as platforms for the display of foreign epitopes on their surfaces in a modular architecture. Depending on the physicochemical properties of the antigenic modules, modularization may affect the expression, solubility and stability of capsomeres, and VLP assembly. In this study, three module designs of a rotavirus hydrophobic peptide (RV10) were synthesized using synthetic biology. Among the three synthetic modules, modularization of the murine polyomavirus VP1 with a single copy of RV10 flanked by long linkers and charged residues resulted in the expression of stable modular capsomeres. Further employing the approach of module titration of RV10 modules on each capsomere via *E. coli* co-expression of unmodified VP1 and modular VP1-RV10 successfully translated purified modular capomeres into modular VLPs when assembled *in vitro*. Our results demonstrate that tailoring the physicochemical properties of modules to enhance modular capsomeres stability is achievable through synthetic biology designs. Combined with module titration strategy to avoid steric hindrance to inter-capsomere interactions, this allows bioprocessing of bacterially-produced *in vitro* assembled modular VLPs.

Keywords: rotavirus, synthetic biology, linkers, module titration, co-expression, *Escherichia coli*

4.1. Introduction

Virus-like particles (VLPs) are highly organized multimeric protein complexes that self-assemble from viral structural protein(s) without the viral genome. VLPs are undergoing research as tools for vaccination, gene therapy, drug delivery, diagnostics, nanomaterials and immune therapy [1,2]. Particularly, their application in the field of vaccinology increased interest following the successful development and approval of three VLP-based vaccines for human use against hepatitis B virus

infection [3,4], human papillomavirus-induced cervical cancer [5] and hepatitis E virus infection [6]. Numerous other VLP-based vaccines against many infectious diseases have shown promising results under pre-clinical and clinical studies [7].

VLP subunits, termed capsomeres, have also gained attention for their potential as alternative second-generation cheap vaccines to VLPs [8-13]. A human papillomavirus 16/18 L1 capsomere vaccine is currently undergoing a phase II clinical trial [14]. Under pre-clinical studies, human papillomavirus L1/L2 capsomeres [11,15-17] and modular murine polyomavirus VP1 capsomeres [8,10,18] have shown promising immunogenicity and protective efficacy results in different animal models.

Both VLPs and capsomeres can serve as platforms for surface display of foreign epitopes or antigens in a modular architecture [8,10]. The murine polyomavirus major capsid protein VP1 has been used for presentation of a single or multiple modules of M2e-peptide epitope from influenza A virus [8,10] and J8i-peptide epitope from group A streptococcus M1 protein [8,19], respectively. These modular architectures were produced using a low-cost *E. coli* expression system and purified in high yields using both chromatographic [8,10,19-21] and non-chromatographic methods [22]. Purified modular VP1 capsomeres can form VLPs via *in vitro* assembly in a cell-free bioreactor [8,23]. This approach reduces VLP manufacturing and processing costs often linked to eukaryotic expression systems [24] and thus enables low-resource countries to use the final product at affordable costs [13]. However, the production of a modular capsomere from *E. coli* and the *in vitro* modular VLP assembly can highly be dependent on the physicochemical properties of the inserted modules. As previously observed, hydrophobic modules at the surface-exposed loop affected the capsomere stability during downstream processing [25,26].

Although nature employs hydrophobicity for its role in protein-protein interactions [27] and in stabilization of proteins [28-30], inserting hydrophobic modules into the surface-exposed loop of viral capsid protein can significantly change protein surface hydrophobicity. The surface hydrophobicity is often associated with changes in protein conformations [31] and related to the ease with which a protein unfolds [32]. Engineering surface-exposed hydrophobic modules is highly desirable to promote correct protein folding during expression and to maintain the solubility and stability of modular capsomeres, thus allowing the formation of modular VLPs via *in vitro* assembly. Insertion of negatively charged residues at both ends of hydrophobic protein domains has shown to enhance the stability of proteins [26,33]. Polar and/or charged amino acids, particularly, glutamic acid, aspartic acid and serine, have contributed most favourably to protein solubility at high net charge [34].

Linkers are the other important elements that have gained considerable success in construct design for the production of proteins [35]. Linkers can improve protein stability [36,37], folding, expression and purification yield [35,38], and for targeting fusion proteins to specific sites *in vivo* to increase their desired biological activity [35]. Linkers are used to separate different moieties of fusion proteins spatially in order to alleviate structural perturbation to moieties [36,39]. More recently, Lua *et al.* [39] designed longer linkers to ensure structural separation and independence between a rotavirus (RV) 18 kDa VP8* antigenic module and the base VLP. Alleviating structural perturbations via incorporation of longer linkers and eliminating steric barrier by VP8* module titration using a baculovirus-insect cell co-expression strategy have facilitated VLP assembly *in vivo*.

In this study, we described three specific module designs containing hydrophobic RV10 peptide from a RV VP8 subunit protein, [40] to address the effect of hydrophobic modules on the stability and *in vitro* assembly of VLPs. In addition, we demonstrated that module titration approach using *E. coli* co-expression strategy is feasible, and necessary for the production of in *vitro* assembled modular VLP displaying hydrophobic RV10. A synthetic biology-based module design and *E. coli* co-expression strategy developed in this study can further enable a rapid and a low-cost processing of modular capsomeres and VLPs presenting modules with adverse physicochemical properties for low-cost vaccine delivery against target diseases at a global scale.

4.2. Materials and methods

4.2.1. Plasmid construction

pGEX-4T-1 plasmid (GE Healthcare Biosciences, Chalfont St. Giles, UK) with inserted murine polyomavirus VP1 sequence (M34958) was generously provided by Professor Robert Garcea (University of Colorado, CO, USA). This construct was designated as GST-VP1 and used for expression of GST-tagged wild-type murine polyomavirus VP1 protein. Plasmid GST-VP1-S4 (Fig. 1A) was generated previously by inserting *Afe*I restriction enzyme site at position 293 of VP1 [8]. Construct GST-ΔVP1 was generated previously by excluding the first 28 amino acids from the N-terminus and the last 63 amino acids from the C-terminus of VP1 sequence, and inserting *Pml*I, *Nae*I, *Afe*I, and *Sna*BI restriction enzyme sites at positions 28 (N-terminus), 85 (loop S1), 293 (loop S4), and 380 (C-terminus) of VP1 positions, respectively [10].

DNA sequences encoding three tandem copies of residues 1-10 (MASLIYRQLL, RV10) from the human RV spike protein VP8 subunit domain [MASLIYRQLLMASLIYRQLLMA SLIYRQLL, $(RV10)_3$], was prepared by annealing of complementary oligos (5´atggcaagcctaatatacagacaactac

taatggcaagcctaatatacagacaactactaatggcaagcctaatatacagacaactacta3´). The prepared gene insert was ligated into *Afe*I-linearized GST-VP1-S4 vector (Fig 1A), and into *Pml*I-, *Afe*I-, or *Sna*BI-linearized GST-ΔVP1 vector (Fig. 1C) sequentially to generate modular constructs VLP-$(RV10)_3$ (Fig. 1B) and Cap$(RV10)_3$ (Fig. 1D), respectively. The gene insert encoding three tandem copies of RV10 with ESE residues inserted between the RV10 peptides, and flanked by E and ES [EMASLIYRQLLESEMASLIYRQLLESEMASLIYRQL LES, $(RV10)_3$ESE] was prepared by annealing complementary oligos (5´gagatggcgagcctcatctatcgccaactcctcgaaagcgaaatggcctctctgatcta ccgccagctgctggagtctgaaatggcgtccctgatttaccgtcaactgctcgaatcc3´). The prepared gene insert was cloned into *Afe*I-linearized GST-VP1-S4 vector, and into *Pml*I-, *Afe*I-, or *Sna*BI-linearized GST-ΔVP1 vector sequentially to generate modular constructs VLP-$(RV10)_3$ESE (Fig. 1B) and Cap$(RV10)_3$ESE (Fig. 1D), respectively.

Similarly, gene insert encoding E4-RV10-E4, a module comprising RV10 with tetra glutamic acid residues (EEEE or E4), was prepared by assembling a set of oligos (5´tataatgggctggagagttacaa3', 5´gatgaggctcgccatctcttcctcttcgcttcttgtaactctccagcccattata3', 5´agagatggcgagcctcatctatcgccaactcctcg aagaagaggaagcttatgat3´, 5'gccctctccagtgatggacatcataagcttcctcttcttcga3´) generated from DNAWork s (http://helixweb.nih,gov/dnaworks/). Linker sequences, G4S-Q25 (residues GGGGSQGVSDLV GLPNQICLQKTTSTILKP) and P6-G4S (residues PAQCSE-GGGGS), were inserted into the N- and C-terminus of E4-RV10-E4 sequence, respectively, at the DNA level using PCR-based gene assembly, annealing and amplification. The prepared gene insert encoding module, G4S-Q25-E4-RV10-E4-P6-G4S, was cloned into *Afe*I-linearized GST-VP1-S4 vector by homologous recombination to generate construct VLP-RV10 (Fig. 1B).

pETDuet-1 vector with multiple cloning sites (MCS1 and MCS2) was purchased from Novagen (Madison, Wisconsin, USA). VP1 gene was cloned into MCS1 of pETDuet-1 vector between *EcoR*I and *Sal*I restriction sites. This vector was designated as pET-VP1 and used for expression of VP1 without a tag. Another construct, designated as pET-VP1-RV10 (Fig. 1E), was generated to carry both VP1 and VP1-RV10 gene inserts for co-expression of the proteins. The *Nae*I restriction enzyme site was inserted into construct VLP-RV10 at position 86 of VP1 as described previously [39]. The VP1-RV10 gene insert with RV10 flanking linkers G4S-Q25-E4 and E4-P6-G4S was amplified from construct VLP-RV10 and cloned into MSC2 of pET-VP1 construct between *Ned*I and *Pac*I restriction sites, to generate construct pET-VP1-RV10. All cloned constructs were verified by DNA sequencing at the Australian Genome Research Facility (Brisbane, Australia).

4.2.2. Protein expression

Constructs GST-VP1, GST-ΔVP1, VLP-$(RV10)_3$, VLP-$(RV10)_3$ESE, VLP-RV10, Cap$(RV10)_3$ and Cap$(RV10)_3$ESE were transformed into *E. coli* Rosetta^{TM}(DE3) pLysS chemically competent cells (Novagen, San Diego, CA, USA), separately. Bacterial expression of all GST-tagged proteins were as previously described [20,21], except cultures were induced with 0.1 mM IPTG (Astra Scientific Pty. Ltd., Gymea NSW, Australia) at 20 ºC for 16 h. Constructs pET-VP1 and pET-VP1-RV10 were transformed into chemically competent *E. coli* Rosetta 2 (DE3) cells (Novagen, San Diego, CA, USA). The transformed cells were grown separately at 37 ºC to an OD_{600} of 0.5 using Luria Bertani (LB) broth containing 50 µg mL^{-1} ampicillin (aMResco®, Solon Ohio, USA) and 34 µg mL^{-1} chloramphenicol (Astral Scientific Pty. Ltd., Gymea NSW, Australia). Expression of non-tagged VP1 and co-expression of VP1 and modular VP1-RV10 were induced with 0.5 mM IPTG at 26 ºC for 16 h. The expression level and solubility of the target proteins were detected using SDS-PAGE that was performed using 10% gel as reported previously [41].

4.2.3. Purification of capsomeres from GST-tagged constructs

Purification of GST-tagged proteins was performed as described previously for purification of GST-tagged wild-type VP1 [20,21,42]. Release of GST from GST-tagged wild-type VP1 (GST-VP1) using thrombin-mediated cleavage and VP1 capsomere purification was carried out as previously reported [10,42]. Tobacco etch virus protease (TEVp) was produced from a recombinant *E. coli* and purified by immobilized metal ion affinity chromatography as reported previously [43]. The pure enzyme was used for release of GST from all other GST-tagged proteins. TEVp-mediated release of the tag protein was performed at 25:1 ratio (w/w) for 2 h at room temperature. The cleavage products were centrifuged (22,000 *g*, 5 min, 4ºC) and the capsomeres were recovered from 1.0 mL of supernatants for each protein with a Superdex 200 10/300 GL column (GE Healthcare Biosciences) operated with an AKTAexplorer^{TM}10 (GE Healthcare Biosciences) liquid chromatography system. The column was pre-equilibrated with L-buffer [40 mM Tris (pH 8.5), 500 mM NaCl, 1mM ethylenediaminetetra-acetic acid (EDTA) disodium, 5% (v/v) glycerol and 5 mM dithiothreitol (DTT)] at a flow rate of 0.5 mL min^{-1}. The column was also pre-equilibrated with L-buffer containing 0.5% (v/v) triton x-100 (TX-100) at a flow rate of 0.5 mL min^{-1} for purification of VLP-$(RV10)_3$ESE capsomeres after TEVp-mediated release of the GST tag in the presence of 0.05% (v/v) TX-100. The Unicorn software (Version 7.0) (GE Healthcare) was used for monitoring sample runs and for analysis and evaluation of the chromatogram data. Target proteins were detected with SDS-PAGE in elution fractions corresponding to the aggregate and capsomere peak fractions of SEC chromatograms.

4.2.4. Purification of capsomeres from non-tagged constructs

Soluble cell lysates were prepared as described previously [20,21,42], except cell pellets were resuspended in L-buffer containing 500 mM NaCl. Capsomeres from constructs pET-VP1 and pET-VP1-RV10 were purified from soluble cell lysate by selective salting-out precipitation for 2 h at 4 ºC using 1 M Na_2SO_4. The protein pellets were collected by centrifugation (22,000 *g*, 5 min at 4 ºC) and resuspended in L-buffer. The soluble portion of the resuspension was separated from the insoluble fraction by centrifugation (22,000 *g*, 5 min at 4 ºC). The capsomeres were further purified by SEC through a Superdex 200 10/300 GL column that was pre-equilibrated with L-buffer at a flow rate of 0.5 mL min^{-1}.

4.2.5. Static light scattering

Molecular weight (MW) of modular capsomere VLP-RV10 was estimated on multi-angled detectors DAWN®EOS™ (Wyatt Technology Corporation) light scattering instrument connected to a high-performance liquid chromatography system (Agilent Technology System). The molecular weight of VP1 capsomeres was estimated under the same conditions as a control. Protein solutions of VP1 and VLP-RV10 (V = 100 µL, C = 10.0-15.0 mg mL^{-1}) after release of GST by enzyme-mediated cleavage were loaded on a Superdex 200 10/300 GL column and eluted with L-buffer at a flow rate of 0.5 mL min^{-1}. The 90º light scattering and UV absorbance at 280 nm of the eluting materials were recorded on a computer and analyzed with the Astra® software supplied by Wyatt Technology Corporation. The 90º light scattering detector was calibrated using bovine serum albumin [100 µL at 2.0 mg mL^{-1}, MW: 66 kDa] as a standard.

4.2.6. VLP assembly and characterization

In vitro assembly of stable modular capsomeres into modular VLPs was as previously described [8], except dialysis was performed for 24 h at room temperature and for 16 h at 4 ºC against assembly buffer GL1 and PBS, respectively. Assembled products were analysed using Asymmetric Flow Field-Flow Fractionation (AF4) coupled with multi-angle light scattering as described previously [44]. Visualization of VLPs with transmission electron microscope (TEM) from *in vitro* assembly was performed as previously reported [44].

4.3. Results and discussion

4.3.1. Module designs

Murine polyomavirus VP1 was engineered to present heterologous antigenic modules on its surface loops [8]. The surface loop S4, position 293 of VP1, contains an *Afe*I site to allow molecular insertion of foreign sequences (Fig. 4-1A). This is a VLP forming platform. For the capsomere platform (Fig. 4-1C), the first 28 and the last 63 amino acid residues of VP1 were excluded. ΔVP1 contains engineered *Pml*I-, *Nae*I-, *Afe*I- and *SnaB*I-restriction enzyme sites at positions 28 (N-terminus), 86 (S1 loop), 293 (S4 loop) and 380 (C-terminus) of VP1 positions, respectively, for insertion of foreign modules. Previous studies have demonstrated good tolerance of heterologous peptide module insertions on both VLP and capsomere platforms [8,10,19]. The physicochemical properties of modules for insertion, such as hydrophobicity and charge, can affect the expression, solubility and stability of modular proteins [25,26].

The RV10 peptide comprises amino acid residues 1-10 of the RV VP8 subunit protein, and is one of the linear B-cell epitopes involved in human RV neutralization [40,45]. RV10, a hydrophobic peptide, is a highly conserved epitope among human and animal RV strains [40]. In this work, three different designs of module RV10 [$(RV10)_3$, $(RV10)_3$ESE and G4S-Q25-E4-RV10-E4-P6-G4S] were synthesized at the DNA level using synthetic biology strategies, with the aim to generate modular constructs for production of stable modular capsomeres and VLPs.

$(RV10)_3$ is a module containing three tandem copies of RV10. Insertion of $(RV10)_3$ at the DNA level into the VLP and capsomere platforms produced constructs VLP-$(RV10)_3$ and Cap$(RV10)_3$, as illustrated in Fig. 4-1. Module $(RV10)_3$ESE contains three tandem copies of RV10 with additional E, ESE and ES residues. Constructs VLP-$(RV10)_3$ESE (Fig. 4-1B) and Cap$(RV10)_3$ESE (Fig. 4-1D) were designed to investigate if the addition of polar and/or charged amino acids to the element $(RV10)_3$ can reduce module hydrophobicity, thus enhance the expression of soluble modular proteins. Previous studies have demonstrated that substitution or incorporation of polar and/or charged amino acids into the sequence of surface exposed residues contribute most favourably to protein solubility [34,46,47] and suppress protein aggregation [26,33].

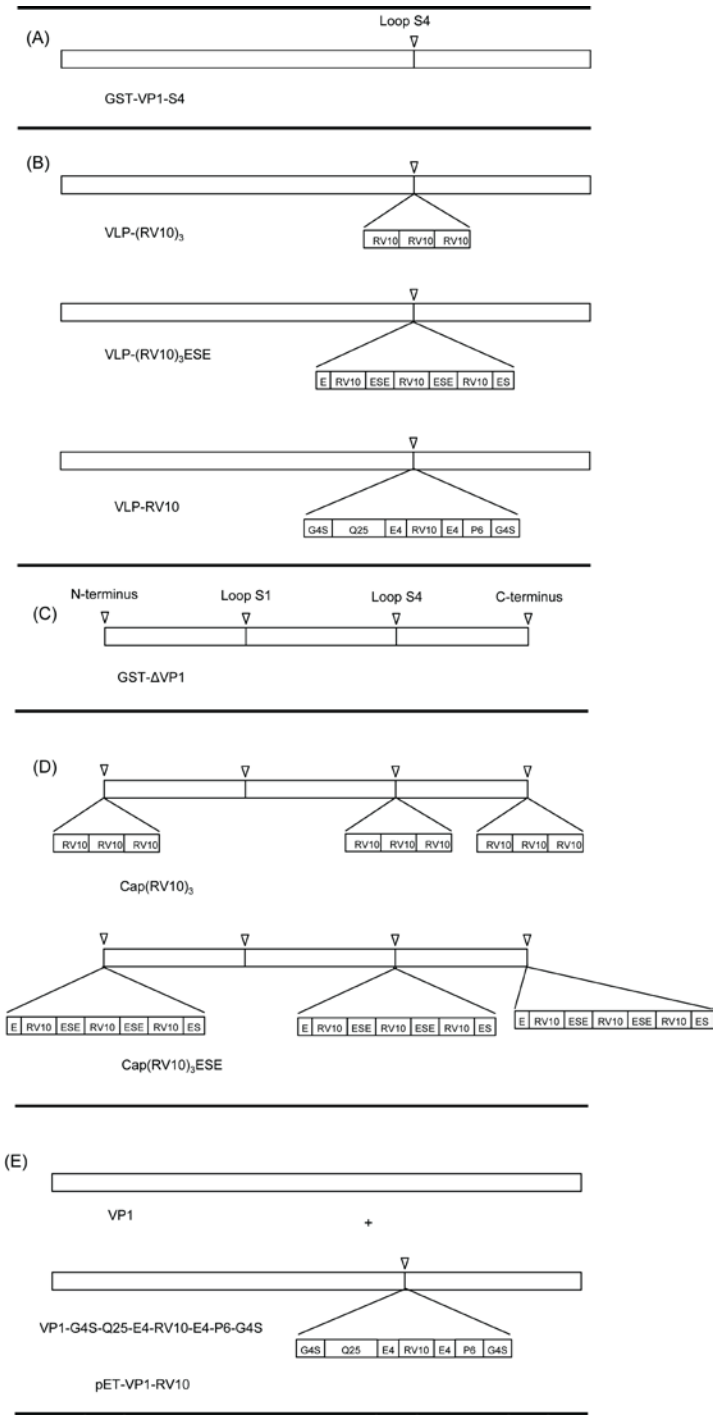

Figure 4-1. Construct designs for modular capsomeres. (A) VLP platform with engineered insertion site at VP1 surface-exposed S4 loop [8], with VP1 protein expressed as GST fusion protein. (B) Modular constructs VLP-$(RV10)_3$, VLP-$(RV10)_3$ESE and VLP-RV10 with inserted modules $(RV10)_3$, $(RV10)_3$ESE and G4S-Q25-E4-RV10-E4-P6-G4S at S4 loop of VP1, respectively. (C) Capsomere platform with engineered N-terminus, S1 loop, S4 loop and C-terminus insertion sites on truncated VP1 [10], expressed as GST fusion protein. (D) Modular constructs Cap$(RV10)_3$ and Cap$(RV10)_3$ESE with inserted modules $(RV10)_3$ and $(RV10)_3$ESE, respectively, at N-terminus, S4 loop and C-terminus insertion sites of truncated VP1. (E) Dual expression construct, pET-VP1-RV10, carrying both wild-type VP1 and modular VP1-G4S-Q25-E4-RV10-E4-P6-G4S sequence.

Module G4S-Q25-E4-RV10-E4-P6-G4S was synthesized by incorporating a single element of RV10 and ionic linkers to shorten the hydrophobic stretch in comparison to $(RV10)_3$ and $(RV10)_3$ESE. Insertion of G4S-Q25-E4-RV10-E4-P6-G4S into the VLP platform at the DNA level produced construct VLP-RV10 (Fig. 4-1B). Longer flanking linkers, G4S-Q25 and G4S-P6, were used for spatial separation of the module from the carrier to alleviate potential structural perturbation, as observed in another study displaying VP8* protein domain on VLP [39].

4.3.2. Module density

Fig. 4-1E illustrates the construct pET-VP1-RV10 designed for dual expression of unmodified VP1 and modular VP1 inserted with G4S-Q25-E4-RV10-P6-G4S module, in *E. coli* co-expression. High surface density of G4S-Q25-E4-RV10-E4-P6-G4S modules on each capsomere (5 copies per capsomere) may cause a steric barrier to inter-capsomere interactions, resulting in poor VLP assembly. This construct was designed to allow titration of module density on the surface of capsomere by co-expressing both VP1 and modular VP1 in a single bacterial cell. The resulting capsomeres may contain a mixture of 5VP1, 4VP1:1VP1-RV10, 3VP1:2VP1-RV10, 2VP1:3VP1-RV10, 1VP1:4VP1-RV10 and 5VP1-RV10. We hypothesized stable capsomeres with reduced module density will lead to *in vitro* assembled VLPs. This co-expression strategy for the reduction of module density on the surface of carrier protein is adopted from the success of a baculovirus-insect cell co-expression system in reducing the surface density of VP8* module to avoid steric barrier to VLP formation [39].

4.3.3. Effect of module hydrophobicity and charge

The expression and solubility of glutathione-S-transferase (GST) fusion modular proteins in comparison with GST fusion VP1 and ΔVP1 were analyzed using SDS-PAGE (Fig. 4-2). No basal expression of the target GST fusion proteins was observed from the cultures harvested before induction with isopropyl-β-D-thiogalactoside (IPTG). The expression and solubility of GST fusion VP1 and ΔVP1 were as previously reported [8,10]. VLP-$(RV10)_3$ was poorly expressed and no expression was detected for Cap$(RV10)_3$. Modularization of the hydrophobic $(RV10)_3$ modules likely increased the hydrophobicity of the modular proteins, thus resulting in poor or no target protein expression. Low level expression in *E. coli* was previously reported for a protein with its highly hydrophobic characteristics [48]. Highly hydrophobic proteins may have adverse effect on host cells, thus prematurely terminated polypeptides, trapped folding intermediates and partially folded proteins may be consistently targeted for degradation to avoid their accumulation in cells [48,49].

The addition of charge residues to RV10 element resulted in soluble expression of modular construct VLP-$(RV10)_3$ESE (Fig. 4-2, Lane 10). Total expression level of modular VLP-$(RV10)_3$ESE and Cap$(RV10)_3$ESE improved significantly in comparison to VLP-$(RV10)_3$ and Cap$(RV10)_3$ (Fig. 4-2). Addition of ESE, E and ES residues to $(RV10)_3$ has enhanced the total protein expression, consistent with previous studies [26,33,34,46,47].

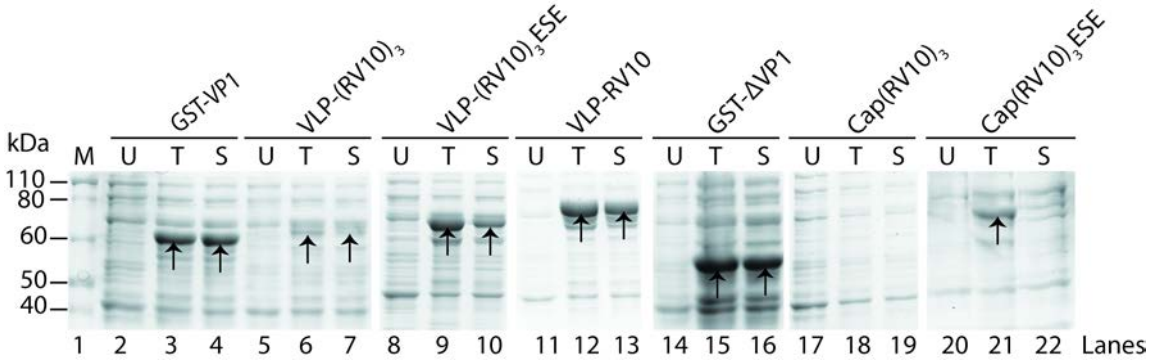

Figure 4-2. Expression and protein solubility of GST fusion target proteins. The target proteins were detected and visualized by SDS-PAGE from un-induced cultures (U), total cell lysate (T) and soluble fraction (S) of induced cultures. Novex® Sharp Pre-stained Protein marker (M) was used as a ladder. Arrows indicate the GST-tagged target proteins.

GST-tagged VLP-$(RV10)_3$ESE was first purified with GST affinity chromatography, followed by TEVp-mediated release of the GST tag and purification of capsomeres by SEC. Both SEC chromatograms (Fig. 4-3A) and SDS-PAGE analysis (Fig. 4-3B) show that mostly soluble aggregates were obtained post removal of GST tag from construct VLP-$(RV10)_3$ESE. This suggests that VLP-$(RV10)_3$ESE modular capsomeres were not stable and prone to aggregation in L-buffer. Modular VLP-$(RV10)_3$ESE capsomeres purified in the presence of TX-100 as stabilizing additive in L-buffer did not form modular VLPs via *in vitro* assembly (Fig. 4-4B). When analyzed on AF4 post VLP assembly, only unassembled capsomeres and soluble aggregates were detected. The specific causes preventing modular VLP assembly for construct VLP-$(RV10)_3$ESE are unknown. One possibility is the hydrophobic-hydrophobic interactions between surface exposed RV10 elements, driving the formation of aggregates. Poor capsomere and VLP stability can also arise due to module insertion, causing a reduction of stabilization energy [50]. Thus, this necessitates specific design of RV10 modules that will maintain capsomeres stability in compatible VLP assembly buffer.

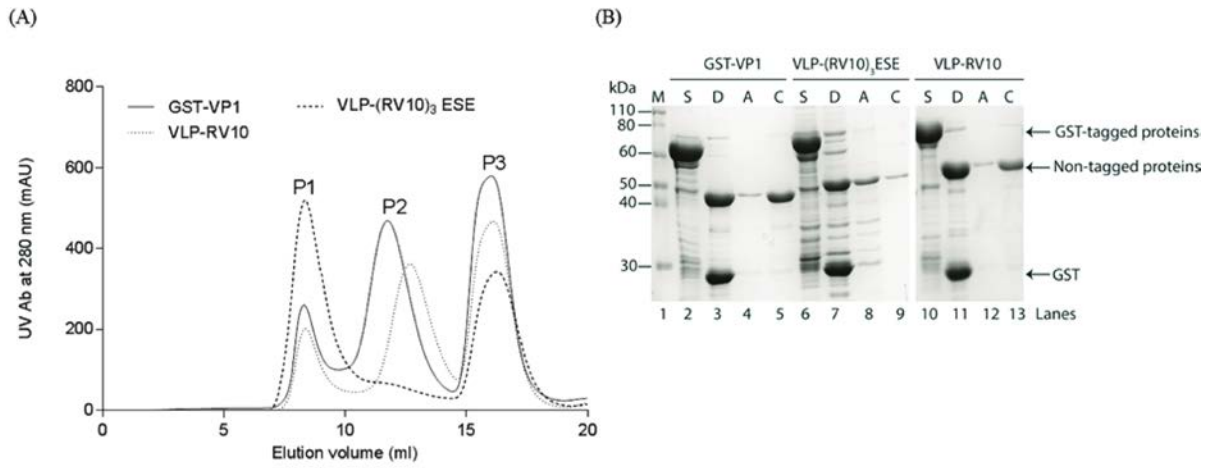

Figure 4-3. Downstream processing of capsomeres. (A) Size exclusion chromatograms of modular capsomeres following TEVp-mediated release of GST tag. P1, P2 and P3 represent the aggregate, capsomere and GST peaks, respectively. (B) SDS-PAGE analysis on downstream processing of capsomeres. Marker protein (M), GST-tagged soluble protein after GST affinity purification (S), TEVp-mediated tag cleavage (D), P1 aggregate peak fraction (A) and P2 capsomere peak fraction (C).

4.3.4. Flanking linker and charged element for surface display of hydrophobic peptide

Construct VLP-RV10 contains module G4S-Q25-E4-RV10-P6-G4S and was expressed as soluble GST fusion target proteins (Fig. 4-2B, Lane 13). Using a single copy of RV10 element with additional surface charged E4 residues likely decreased the hydrophobicity of modular proteins and resulted in good expression level of soluble proteins. Previously, insertion of E4 residues as ionic flanking elements has enhanced expression of soluble modular VP1 containing a hydrophobic H190 module from influenza virus [51]. Studies have demonstrated positive correlation of decreasing hydrophobicity with high protein expression and solubility levels [52,53].

In addition to high level of soluble proteins with construct VLP-RV10, stable capsomeres were obtained post release of GST tag (Fig. 4-3). The SEC chromatogram suggests a more compact capsomere structure for VLP-RV10 as the capsomeres eluted later than unmodified VP1 capsomeres. The MW of VP1 and modular VLP-RV10 capsomeres analyzed by static light scattering were 219.2 kDa and 270.2 kDa, respectively, confirming their pentameric structure forms (Fig. 4-4A). Despite obtaining stable VLP-RV10 capsomeres, no VLP was detected after *in vitro* VLP assembly (Fig. 4-4B).

Decreasing module hydrophobicity by reducing elements of RV10 in a module can minimize hydrophobic interaction forces that drive protein aggregation [54]. The E4 ionic linkers increased the surface charge of the module; consequently the electrostatic repulsive forces between protein molecules can counteract the hydrophobic interaction forces, enhancing the stability of modular

capsomeres. Introduction of longer flanking linkers G4S-Q25 and P6-G4S might have alleviated structural perturbation of capsomeres. We speculate that the lack of VLP assembly is caused by either strong electrostatic-repulsive interactions or steric hindrance between modular capsomeres due to high module density on capsomere surface.

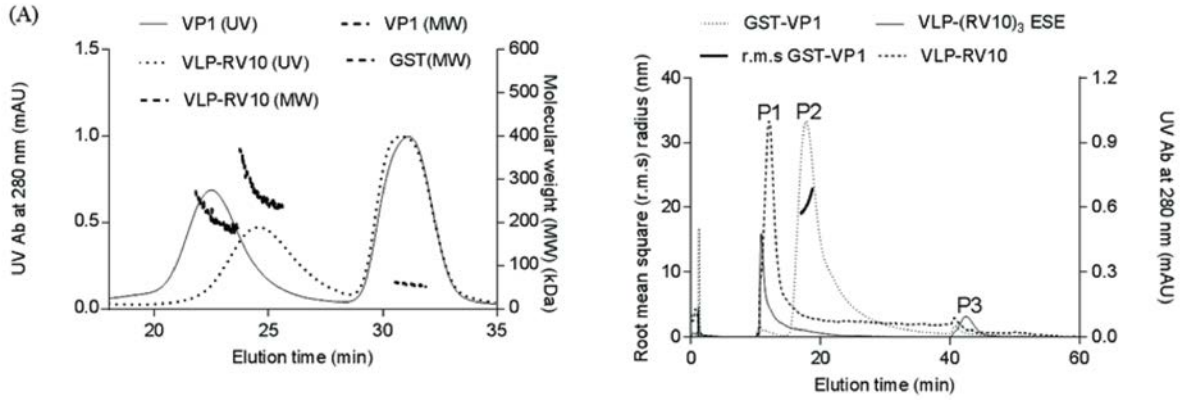

Figure 4-4. Characterization of capsomeres and in vitro assembled products. (A) SEC-HPLC/LS analysis of VP1 and VLP-RV10 capsomeres following TEVp-mediated release of GST tag. P1, P2 and P3 represent peaks for VP1 capsomeres, VLP-RV10 capsomeres and GST dimers, respectively. (B) AF4 fractograms of in vitro assembled products of VP1, VLP-(RV10)$_3$ESE and VLP-RV10. P1, P2 and P3 represent peaks for non-assembled proteins, assembled VLPs and aggregates, respectively.

4.3.5. Module titration for *in vitro* assembly of VLP

As shown in Fig. 4-5A, soluble VP1 and VP1-RV10 proteins were expressed in *E. coli* without GST tag. When both proteins were co-expressed in a dual expression system, VP1-RV10 was expressed at higher level than VP1. Co-expression of two or more genes from a single expression vector might lead to imbalance ratio of expressed proteins due to differences in the rates of translation [55], transcription, translocation, and the stability of RNA and protein products [56,57].

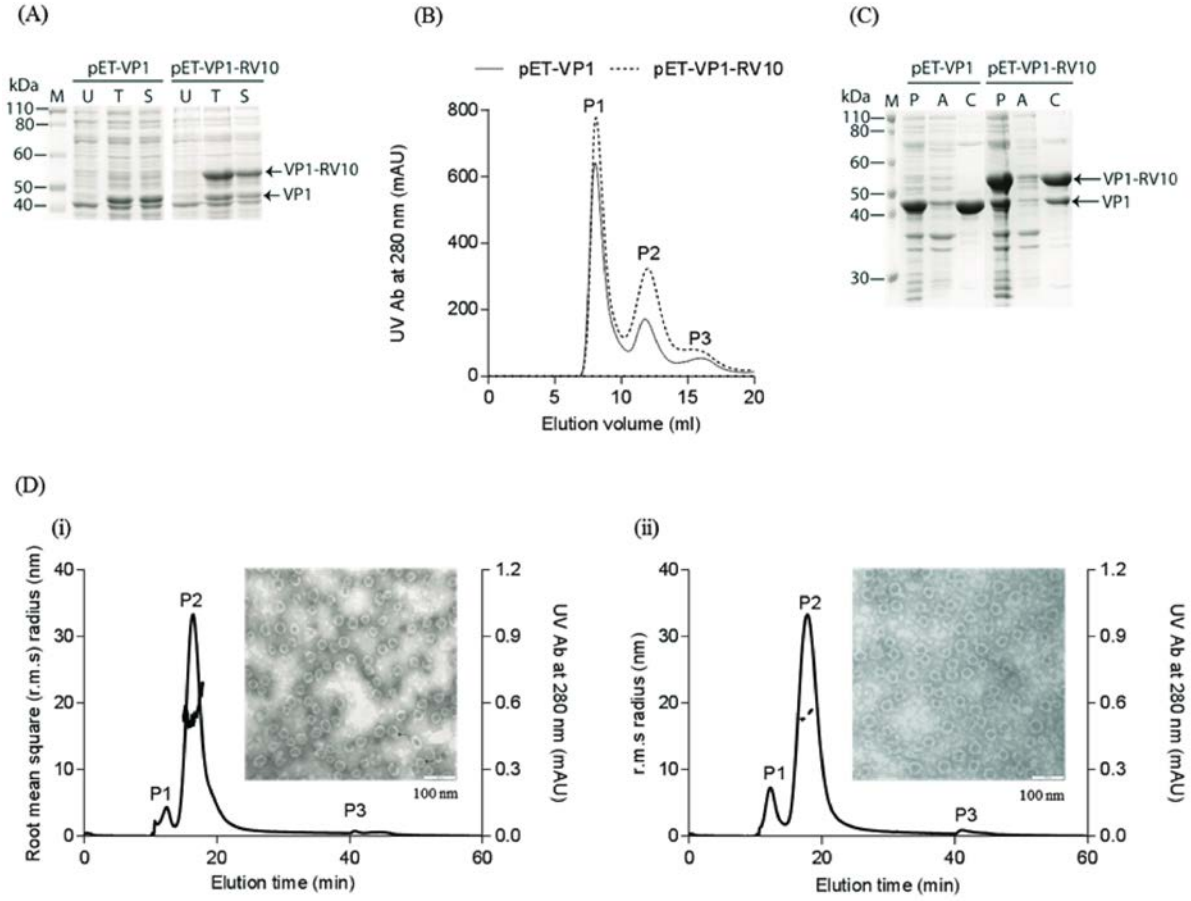

Figure 4-5. Co-expression strategy for bacterially-produced stable modular capsomeres and in vitro assembled modular VLPs presenting module RV10. (A) Detection and visualization of non-tagged target proteins of pET-VP1 and pET-VP1-RV10 from cell lysates. (B) Size exclusion chromatograms of pET-VP1 and pET-VP1-RV10 capsomeres following purification by selective salting-out precipitation. P1, P2 and P3 represent the aggregate, capsomere, and co-precipitated E. coli protein peaks, respectively. (C) SDS-PAGE analysis of the target proteins. Marker protein (M), purified protein by selective salting-out precipitation (P), P1 aggregate peak fraction (A) and P2 capsomere peak fraction (C). (D) AF4 fractograms and TEM micrographs of assembled products (i) VP1VLP and (ii) RV10VLP. P1, P2 and P3 represent peaks for non-assembled proteins, assembled VLPs and aggregates, respectively.

Capsomeres of pET-VP1 (CapVP1) and pET-VP1-RV10 (CapRV10) were purified from clarified cell lysates by selective salting-out precipitation followed by a further polishing SEC step. Fig. 4-5B and 4-5C show that stable modular capsomeres were obtained after purification. Co-precipitated *E. coli* proteins, in the aggregated fractions, were separated from the capsomeres during the SEC polishing step (Fig. 4-5C). After *in vitro* assembly, modular VLPs (RV10VLP) comprising VP1 and VP1-RV10 capsomeres were obtained, as analyzed on AF4 and TEM (Fig. 4-5D). A small peak of unassembled proteins was detected on AF4 with a VLP peak detected at 18 to 21 min elution time. The average root-mean-square (r.m.s) radius for the RV10VLP was 21.4 nm, similar to the size distribution of unmodified VP1VLP (r.m.s radius 22.5 nm). Under TEM, RV10VLP was similar in morphology to the unmodified VP1VLP (Fig. 4-5D). This co-expression strategy of both VP1 and

VP1-RV10 proteins to reduce the RV10 module density per capsomere was a successful approach to obtain stable RV10VLPs. Reducing the density of modules on the surface of capsomeres potentially minimize hydrophobic interactions, electrostatic repulsive interactions and overcome steric hindrance of modules to the formation of modular VLPs. Here, we have demonstrated that co-expression strategy to reduce module density is feasible for bacterially-produced modular capsomeres displaying hydrophobic peptide RV10.

4.4. Conclusion

Using synthetic biology, hydrophobic RV10 peptide epitope of RV VP8 spike protein was modularized on murine polyomavirus VP1 capsid protein. The hydrophobicity of element RV10 deters the tandem copies display strategy to increase the ratio of antigenic module to base protein VP1. Flanking hydrophobic element with charged glutamic acid (E4) can reduce hydrophobicity, to prevent hydrophobic-interaction driven protein aggregation. However, the addition of long flanking linkers (G4S-Q25 and P6-G4S) potentially displays the module away from base protein VP1, thus avoid structural perturbation by the module. The module design G4S-Q25-E4-RV10-E4-P6-G4S yielded high expression of modular proteins and stable modular capsomeres after purification. Attempts to assemble these stable modular capsomeres via *in vitro* VLP assembly were not successful. The results suggest that titrating module density down by applying co-expression strategy of both unmodified VP1 and modular VP1 in a single bacterial cell allows an optimal ratio of VP1:VP1-RV10 capsomere formation, which then lead to VLP assembly. Module titration can minimize hydrophobic interactions, electrostatic repulsive interactions or prevent steric barrier to modular VLP assembly. The strategies employed in this study provide a powerful approach for modularization of VLP and capsomere with various hydrophobic antigenic modules, such as T-cell epitopes, that are potentially challenging due to their physiochemical properties. The design strategies can also be used to modulate the hydrophobic domains of other proteins for enhanced expression of soluble and stable proteins using a low-cost prokaryotic expression system.

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References

1. Lua LHL, Connors NK, Sainsbury F, Chuan YP, Wibowo N, Middelberg APJ: **Bioengineering virus like-particles as vaccines**. *Biotechnollogy and Bioengineering* 2014, **111**:425-440.
2. Zeltins A: **Constructions and characterizations of virus-like particles: a review**. *Molecular Biotechnology* 2012, **53**:92-107.
3. Adkins J, AJ W: **Recombinant hepatitis B vaccine: a review of its immunogenicity and protective efficacy against hepatitis B**. *Biodrugs* 1998, **10**:137-158.
4. Keating G, Noble S: **Recombinant hepatitis B vaccine (Engerix-B): a review of its immunogenicity and protective efficacy against hepatitis B**. *Drugs* 2003, **63**:1021-1051.
5. Siddiqui M, Perry C: **Human papillomavirus quadrivalent (types 6, 11, 16, 18) recombinant vaccine (Gardasil)**. *Drugs* 2006, **66**:1263-1271.
6. Wu T, Li SW, Zhang J, Ng MH, Xia NS, Zhao Q: **Hepatitis E vaccine development: a 14 year odyssey**. *Human Vaccines and Immunotherapeutics* 2012, **8**:823-827.
7. Roldao A, Mellado M, Castilho L, Carrondo M, Alves P: **Virus-like particles in vaccine development**. *Expert Review Vaccines* 2010, **9**:1149-1176.
8. Middelberg APJ, Rivera-Hernandez T, Wibowo N, Lua LHL, Fan Y, Magor G, Chang C, Chuan YP, Good MF, Batzloff MR: **A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines**. *Vaccine* 2011, **29**:7154-7162.
9. Schädlich L, Senger T, Kirschning CJ, Müller M, Gissmann L: **Refining HPV 16 L1 purification from *Escherichia coli*: Reducing endotoxin contaminations and their impact on immunogenicity**. *Vaccine* 2009, **27**:1511-1522.
10. Wibowo N, Chuan YP, Lua LHL, Middelberg APJ: **Modular engineering of a microbially-produced viral capsomere vaccine for influenza**. *Chemical Engineering Science* 2012, **103**:12-20.
11. Dell K, Koesters R, Linnebacher M, Klein C, Gissmann L: **Intranasal immunization with human papillomavirus type 16 capsomeres in the presence of non-toxic cholera toxin-based adjuvants elicits increased vaginal immunoglobulin levels**. *Vaccine* 2006, **24**:2238-2247.
12. Senger T, Schädlich L, Textor S, Klein C, Michael KM, Buck CB, Gissmanna L: **Virus-like particles and capsomeres are potent vaccines against cutaneous alpha HPVs**. *Vaccine* 2010, **28**:1583-1593.
13. Chuan YP, Wibowo N, Lua LHL, Middelberg APJ: **The economics of virus-like particles and capsomere vaccines**. *Biochemical Engineering Journal* 2014, **90**:255-263.

14. Kumar SVAS, Biswas BM, Jose CT: **HPV vaccine: current status and future directions**. *Medical Journal Armed Forces India* 2015, **71**:171-177.

15. Yuan H, Estes PA, Chen Y, Newsome J, Olcese VA, Garcea RL, Schlegel R: **Immunization with a pentameric L1 fusion protein protects against papillomavirus infection**. *Journal of Virology* 2001, **75**:7848-7853.

16. Wu WH, Gersch E, Kwak K, Jagu S, Karanam B, Huh WK, Garcea RL, Roden RBS: **Capsomer vaccines protect mice from vaginal challenge with human papillomavirus**. *PloS One* 2011, **6**:e27141.

17. Jagua S, Kwaka K, Robert L. Garceab, Rodena RBS: **Vaccination with multimeric L2 fusion protein and L1 VLP or capsomeres to broaden protection against HPV infection**. *Vaccine* 2010, **28**:4478-4486.

18. Wibowo N, Hughes FK, Fairmaid EJ, Lua LHL, Brown LE, Middelberg APJ: **Protective efficacy of a bacterially produced modular capsomere presenting M2e from influenza: extending the potential of broadly cross-protecting epitopes**. *Vaccine* 2014, **32**:3651-3655.

19. Rivera-Hernandez T, Hartas J, Wu Y, Chuan YP, Lua LHL, Good M, Batzloff MR, Middelberg APJ: **Self-adjuvanting modular virus-like particles for mucosal vaccination against group A streptococcus (GAS)**. *Vaccine* 2013, **31**:1950-1955.

20. Liew MWO, Rajendran A, Middelberg APJ: **Microbial production of virus-like particle vaccine protein at gram-per-litre levels**. *Journal of Biotechnology* 2010, **150**:224-231.

21. Chuan YP, Lua LHL, Middelberg APJ: **High-level expression of soluble viral structural protein in *Escherichia coli***. *Journal of Biotechnology* 2008, **134**:64-71.

22. Wibowo N, Wu Y, Fan Y, Meers J, Lua LHL, Middelberg APJ: **Non-chromatographic preparation of a bacterially produced single-shot modular virus-like particle capsomere vaccinefor avian influenza**. *Vaccine* 2015, **33**:5960-5965.

23. Liew MWO, Chuan YP, Middelberg APJ: **High-yield and scalable cell-free assembly of virus-like particles by dilution**. *Biochemical Engineering Journal* 2012, **67**:88-96.

24. Palkova Z, Adamec T, Liebl D, Stokrova J, Forstova J: **Production of polyomavirus structural protein VP1 in yeast cells and its interaction with cell structures**. *FEBS Letters* 2000, **478**:281-289.

25. Tekewe A, Connors NK, Sainsbury F, Wibowo N, Lua LHL, Middelberg APJ: **A rapid and simple screening method to identify conditions for enhanced stability of modular vaccine candidates**. *Biochemical Engineering Journal* 2015, **100**:50-58.

26. Abidin RS, Lua LHL, Middelberg APJ, Sainsbury F: **Insert engineering and solubility screening improves recovery of virus-like particle subunits displaying hydrophobic epitopes**. *Protein Science* 2015, **24**:1820-1828.

27. Chanphai P, Bekale L, Tajmir-Riahi HA: **Effect of hydrophobicity on protein-protein interactions**. *European Polymer Journal* 2015, **67**:224-231.

28. Fagain C: **Understanding and increasing protein stability**. *Biochimica et Biophysica Acta* 1995, **1252**:1-14.

29. Pace CN: **Contribution of the hydrophobic effect to globular protein stability**. *Journal of Molecular Biology* 1992, **226**:29-35.

30. Pace CN, Fu H, Fryar KL, Landua J, Trevino SR, Shirley BA, Hendricks MM, Iimura S, Gajiwala K, Scholtz JM, et al.: **Contribution of hydrophobic interactions to protein stability**. *Journal of Molecular Biology* 2011, **408**:514-528.

31. Chao CC, Yan-Shan M, Stadtman ER: **Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems**. *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**:2969-2974.

32. Mann DF, Shah K, Stein D, Snead GA: **Protein hydrophobicity and stability support the thermodynamic theory of protein degradation**. *Biochimica et Biophysica Acta* 1984, **788**:17-22.

33. Perchiacca JM, Ladiwala AR, Bhattacharya M, Tessier PM: **Aggregation-resistant domain antibodies engineered with charged mutations near the edges of the complementarity-determining regions**. *Protein Engineering Design and Selection* 2012, **25**:591-601.

34. Trevino SR, Scholtz JM, Pace CN: **Amino acid contribution to protein stability: Asp, Glu and Ser contribute more favorably than the other hydrophilic amino acids in RNase Sa**. *Journal Molecular Biology* 2007, **366**:449-460.

35. Chen X, Zaro JL, Shen WC: **Fusion protein linkers: property, design and functionality**. *Advanced Drug Delivery Reviews* 2013, **65**:1357-1369.

36. Zhao HL, Yao XQ, Xue C, Wang Y, Xiong XH, Liu ZM: **Increasing the homogeneity, stability and activity of human serum albumin and interferon-α2b fusion protein by linker engineering**. *Protein Expression and Purification* 2008, **61**:73-77.

37. Hao HJ, Jiang YQ, Zheng YL, Ma R, Yu DW: **Improved stability and yield of Fv targeted superantigen by introducing both linker and disulfide bond into the targeting moiety**. *Biochimie* 2005, **87**:661-667.

38. Klement M, Liu C, Loo BLW, Choo ABH, Siak-Wei Ow D, Lee DY: **Effect of linker flexibility and length on the functionality of a cytotoxic engineered antibody fragment**. *Journal of Biotechnology* 2015, **199**:90-97.

39. Lua LHL, Fan Y, Chang C, Connors NK, Middelberg APJ: **Synthetic biology design to display an 18 kDa rotavirus large antigen on a modular virus-like particle** *Vaccine* 2015, **33**:5937-5944.

40. Kovacs-Nolan J, Yoo DW, Mine Y: **Fine mapping of sequential neutralization epitopes on the subunit protein VP8 of human rotavirus**. *Biochemical Journal* 2003, **376**:269-275.

41. Laemmli U: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4**. *Nature* 1970, **227**:680-685.

42. Lipin DL, Lua LHL, Middelberg APJ: **Quaternary size distribution of soluble aggregates of glutathione-S-transferase purified viral protein as determined by asymmetrical flow field flow fractionation and dynamic light scattering**. *Journal of Chromatography A* 2008, **1190**:204-214.

43. Cabrita LD, Gilis D, Robertson AL, Dehouck Y, Rooman M, Bottomley SP: **Enhancing the stability and solubility of TEV protease using in silico design**. *Protein Science* 2007, **16**:2360-2367.

44. Chuan YP, Fan Y, Lua LHL, Middelberg APJ: **Quantitative analysis of virus-like particle size and distribution by field-flow fractionation**. *Biotechnology and Bioengineering* 2008, **99**:1425-1433.

45. Kovacs-Nolan J, Mine Y: **Tandem copies of a human rotavirus VP8 epitope can induce specific neutralizing antibodies in BALB/c mice**. *Biochimica et Biophysica Acta* 2006, **1760**:1884-1893.

46. Vernet E, Kotzsch A, Voldborg B, Sundsstrom M: **Screening of genetic parameters for soluble protein expression in *Escherichia coli***. *Protein Expression and Purification* 2011, **77**:104-111.

47. Pokkuluri PR, Raffen R, Dieckman L, Boogaard C, Stevens FJ, Schiffer M: **Increasing protein stability by polar surface residues: domain-wide consequences of interactions within a loop**. *Biophysic Journal* 2002, **82**:391-398.

48. Ciccaglione A, Marcantonio C, Costantino A, Equestre M, Geraci A, Rapicetta M: **Expression and membrane association of hepatitis C virus envelope 1 protein**. *Virus Genes* 2000, **21**:223-226.

49. Niiranen L, Espelid S, Karlsen CR, Mustonen M, Paulsen SM, Heikinheimo P, Willassen NP: **Comparative expression study to increase the solubility of cold adapted *Vibrio* proteins in *Escherichia coli***. *Protein Expression Purification* 2007, **52**:210-218.

50. Zhang L, Tang R, Bai S, Connors NK, Lua LHL, Chuan YP, Middelberg APJ, Sun Y: **Energetic changes caused by antigenic module insertion in a virus-like particle revealed by experiment and molecular dynamic simulations**. *PloS One* 2014, **9**:e107313.

51. Anggraeni MR: **Engineering of virus-like particles for alternative vaccine candidate targeting hyper-variable peptide antigen-element. PhD Thesis, The University of Queensland. DOI: 10.14264/uql.2015.135** 2014.

52. Idicula-Thomas S, Balaji PV: **Understanding the relationship between the primary structure of proteins and its propensity to be soluble on overexpression in *Escherichia coli***. *Protein Sci* 2004, **14**:582-592.

53. Price WN, Handelman SK, Everett JK, Tong SN, Bracic A, Luff JD, Naumov V, Acton T, Manor P, Xiao R, et al.: **Large-scale experimental studies show unexpected amino acid effects on protein expression and solubility *in vivo in Escherichia coli***. *Microb Inform Exp* 2011, **1**:DOI: 10.1186/2042-5783-1181-1186.

54. Valerio M, Colosimo A, Conti F, Giuliani A, Grottesi A, Manetti C, Zbilut JP: **Early events in protein aggregation: molecular flexibility and hydrophobicity/charge interaction in amyloid peptides as studied by molecular dynamics simulations**. *Proteins: Structure, Function, and Bioinformatics* 2005, **58**:110-118.

55. Tsao K, Waugh D: **Balancing the production of two recombinant proteins in *Escherichia coli* by manipulating plasmid copy number: high-level expression of heterodimeric Ras farnesyltransferase**. *Protein Expression and Purification* 1997, **11**:233-240.

56. Kerrigan JJ, Xie Q, Ames RS, Lu Q: **Production of protein complexes via co-expression**. *Protein Expression and Purification* 2011, **75**:1-14.

57. Karamitros CS, Konrad M: **Bacterial co-expression of the α and β protomers of human L-asparaginase-3: achieving essential N-terminal exposure of a catalytically critical threonine located in the β-subunit**. *Protein Expression and Purification* 2014, **93**:1-10.

Chapter 5

Integrated molecular and bioprocess engineering for bacterially-produced immunogenic modular virus-like particle vaccine displaying 18 kDa rotavirus antigen

The entire Chapter 5 consists of the journal article submitted as:

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The following modifications were made to the article:

- Page numbers of the original article were changed into the numbers consistent with those on the remainder of the thesis page numbers.
- Figure numbers were changed into the numbers consistent with those on the remainder of figure in the thesis.
- Section and sub-section numbers were changed into the numbers consistent with those on the remainder of section and subsection numbers in all chapters of the thesis.
- The reference style of the original article was changed into the style consistently used in all chapters of the thesis.

Integrated molecular and bioprocess engineering for bacterially-produced immunogenic modular virus-like particle vaccine displaying 18 kDa rotavirus antigen

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Abstract

A high global burden of rotavirus disease and the unresolved challenges with the marketed rotavirus vaccines, particularly in the developing world, have ignited efforts to develop virus-like particle (VLP) vaccines for rotavirus. While rotavirus-like particles comprising multiple viral proteins can be difficult to process, modular VLPs presenting rotavirus antigenic modules are promising alternatives in reducing process complexity and cost. In this study, integrated molecular and bioprocess engineering approaches were used to simplify the production of modular murine polyomavirus capsomeres and VLPs presenting a rotavirus 18 kDa VP8* antigen. A single construct was generated for dual expression of non-tagged murine polyomavirus capsid protein VP1 and modular VP1 inserted with VP8*, for co-expression in *Escherichia coli*. Co-expressed proteins assembled into pentameric capsomeres in *E. coli*. A selective salting-out precipitation and a polishing size exclusion chromatography step allowed the recovery of stable modular capsomeres from cell lysates at high purity, and modular capsomeres were successfully translated into modular VLPs when assembled *in vitro*. Immunogenicity study in mice showed that modular capsomeres and VLPs induced high levels of VP8*-specific antibodies. Our results demonstrate that a multipronged synthetic biology approach combining molecular and bioprocess engineering enabled simple and low-cost production of highly immunogenic modular capsomeres and VLPs presenting conformational VP8* antigenic modules. This strategy potentially provides a cost-effective production route for modular capsomere and VLP vaccines against rotavirus, highly suitable to manufacturing economics for the developing world.

Keywords: Capsomeres, co-expression, selective precipitation, murine polyomavirus, large antigen, *Escherichia coli*

5. 1. Introduction

Rotavirus (RV) is the major enteric pathogen causing severe diarrhoea in children under 5 years of age worldwide [1]. It caused nearly 500,000 deaths of children worldwide in 2008 in which 90% of the deaths occurred in low-income developing countries in Africa and Asia [2]. The situation still remains unacceptably worse in most developing countries [3]. Despite the high mortality and morbidity rate of the disease, no RV-specific drugs are available to date. Limited information on therapeutic targets have impeded efforts for RV-specific drug discovery and development [4]. Thus, vaccination remains the primary prophylactic strategy to reduce the disease burden of RV [5,6].

The use of two live-attenuated oral RV vaccines, RotaTeq® (Merck) and Rotarix™ (GlaxoSmithKline), has reduced childhood mortality rate in developed countries [7,8]. Some developing countries have included the vaccines into their childhood immunization program. However, the vaccines are less effective [9], too costly and logistically prohibitive for use in the developing world [10]. Both Rotarix™ (US$ 2.50 per dose) and RotaTeq® (US$ 3.50 per dose) are still too expensive for low-income developing countries [10]. They also come with possible risk of reversion and intussusception [11]. The high disease burden of RV and the challenges associated with current vaccines, particularly in developing countries, have ignited efforts to develop next-generation low-cost, effective and safe vaccine candidates. These vaccine candidates under development include non-replicating subunits such as synthetic peptides, proteins and virus-like particles (VLPs) [12,13].

Several studies have demonstrated the role of the VP8 subunit domain of RV spike protein and its potential as a vaccine [14-16]. Particularly, the lectin-like domain of VP8 (residues 64-223), known as VP8*, has been shown to induce RV neutralizing antibodies [12,17-20]. However, effectiveness of VP8* is highly dependent on the co-administration of adjuvants that are not approved for use in human vaccination [14,21,22].

Rotavirus-like particles (RLPs) are an effective and potentially safe alternative vaccine candidate to soluble proteins and live-attenuated oral vaccines [23]. They are synthetic mimics of the virus [24], with proven immunogenicity and protection efficacy in animal studies [13,23]. As rotavirus is a complex virus, production of RLPs via co-expression of two or more structural proteins inside eukaryotic cells is also highly complex [25-27]. Process complexity often reduces yield and

increases cost [27]. Consequently, RLPs may be highly expensive vaccine candidates, limiting applicability for use in the developing world.

Minimizing the cost of bioprocessing has become a focus of many developments to produce safe and effective vaccine candidates at affordable cost for use in the developing world [28]. The use of synthetic biology for vaccine design is one of the strategies that have gained attention for addressing manufacturing and processing gaps of vaccine candidates. With the aid of synthetic biology and computational tools [29,30], full length, or truncated proteins or small peptide epitopes have been molecularly inserted into viral structural proteins to generate a new class of bioengineered modular VLP vaccines [31,32]. Insertion of a RV 18 kDa VP8* antigen into the protrusion (P) domain of the norovirus capsid protein produced a highly immunogenic modular VLP subunit vaccine against RVs inside *E. coli* cells [33]. Lua *et al*. (2015) have designed a stable modular murine polyomavirus VLP vaccine candidate presenting VP8* using purposefully designed linkers and module titration via a baculovirus-insect cell co-expression strategy. However, VLP production *in vivo* has limitations on yield and scalability, and the high operating costs associated with VLP recovery from cells [30,33].

An alternative and proven approach for VLP production is cell-free *in vitro* assembly of VLP, which has been developed for low-cost production of highly purified and high quality VLPs using murine polyomavirus VP1 VLP vaccine platform [34,35]. This platform has shown great potential for mass-scale production of highly immunogenic and protective modular VLPs presenting J8-peptide module from group A streptococcus [35,36]. The existing VLP platform uses glutathione-S-transferase (GST) fusion partner as a solubility and affinity tag for the expression and purification of GST fusion VP1 proteins. The use of GST tag comes with major drawbacks, such as poor binding affinity of GST fusion proteins to affinity chromatography media, formation of macromolecular soluble aggregates and high-priced affinity chromatography media [37]. Subsequent enzyme-mediated release of the GST tag also adds complexity and cost to the bioprocess [38,39]. Process complexity and high operating costs due to the use of GST tag rationalized the need for non-tagged protein expression for simple and low-cost recovery of capsomeres for *in vitro* VLP assembly. The concept of tag-free VP1 expression in *E. coli* has been proven with high expression yield of VP1 after optimization of expression parameters [40,41].

The present study describes a combination of molecular and bioprocessing engineering approaches for simplified production of bacterially-produced and *in vitro* assembled modular VLPs presenting a RV 18 kDa VP8* module. A module titration strategy allowed correct presentation of conformational VP8* module on the surface of *E. coli* produced capsomeres and VLPs. In addition,

we demonstrated that expression of non-tagged proteins in *E. coli* and protein recovery using selective salting-out precipitation simplify the bioprocessing of modular capsomeres for subsequent production of *in vitro* assembled modular VLPs with high purity, stability and immunogenicity. The new strategy based on integrated molecular and bioprocess engineering approaches in this study provide an efficient and powerful approach for low-cost production of highly immunogenic modular capsomere and VLP vaccine candidates readied for further translation against RV in the developing world.

5.2. Materials and Methods

5.2.1. Plasmid construction

Murine polyomavirus VP1 sequence (M34958) was cloned into the first multiple cloning sites (MCS1) of pETDuet-1 (Novagen, Madison, Wisconsin, USA) between *Eco*RI and *Sal*I restriction sites. This construct was designated as VP1 and used for the expression of wild-type VP1 proteins. Another construct, designated as VP1-VP8*, was generated to carry both VP1 (at MCS1) and VP1-VP8* (at MCS2) gene inserts for co-expression of the proteins. The VP1-VP8* gene insert with VP8* flanking linkers G4S-Q25 and P6-G4S was amplified from construct pBAC-VP1-Q25-VP8*-P6 [30], and cloned into MSC2 of VP1 construct between *Nde*I and *Pac*I restriction sites. All cloned constructs were verified by DNA sequencing at the Australian Genome Research Facility (Brisbane, Australia).

5.2.2. Protein expression

Constructs VP1 and VP1-VP8* were transformed separately into chemically competent *E. coli* Rosetta 2 (DE3) cells (Novagen, San Diego, CA, USA). VP1 and VP1-VP8* transformed *E. coli* Rosetta 2 (DE3) cells were grown separately at 37 ºC to an OD_{600} of 0.5 using Luria Bertani (LB) broth containing 50 µg mL^{-1} ampicillin (aMResco®, Solon Ohio, USA) and 34 µg mL^{-1} chloramphenicol (Astral Scientific Pty. Ltd., Gymea NSW, Australia). Cultures were induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) (Astra Scientific Pty. Ltd., Gymea NSW, Australia) at 26 ºC for 16 h for protein expression.

5.2.3. Capsomeres purification

Cell pellets from each of 200 mL cultures of VP1 and VP1-VP8* were re-suspended separately in 20 mL L-buffer [40 mM Tris, 500 mM NaCl, 1 mM ethylenediaminetetra-acetic acid (EDTA) disodium, 5% (v/v) glycerol, 5 mM dithiothreitol (DTT), pH 8.5]. Cells were lysed by sonication and cell lysates were clarified by centrifugation as previously described [42]. Target proteins were

precipitated from the cell lysates using 1 M Na_2SO_4 for 2 h at 4 ºC. The precipitates were collected by centrifugation (22,000 *g*, 5 min at 4 ºC) and re-suspended in 2 mL of L-buffer. The soluble portion of the re-suspension was separated from the insoluble fraction by centrifugation (22,000 *g*, 5 min at 4 ºC). VP1 and VP1-VP8* capsomeres, designated wild-type capsomere (wtCap) and CapVP8*, respectively, were recovered from supernatants by size exclusion chromatography (SEC) through a Superdex 200 10/300 GL column (GE Healthcare, UK). The column was pre-equilibrated with either L1-buffer [40 mM Tris, 300 mM, pH 8.5) or L2-buffer [40 mM Tris, 300 mM NaCl, 1 mM EDTA disodium, 5% (v/v) glycerol, 5 mM DTT, pH 8.5] at a flow rate of 0.5 mL min^{-1}. Bacterial endotoxin was removed from capsomere fractions with an anion exchanger as described previously [35].

5.2.4. Characterization and formulation of capsomeres

Qualitative analysis of all protein samples, for detection and visualization of protein purity from capsomere fractions, was performed by SDS-PAGE gel electrophoresis using 10% gel as reported previously [43]. All protein concentrations were determined at 280 nm, based on the Beer-Lambert Law [44]. Theoretical molecular weight and extinction coefficient of each protein was obtained from ProtParam [45]. The concentration of capsomere solutions was adjusted to 1.0 mg mL^{-1} by ultracentrifugal filtration using Amicon®Ultra-4.0 ml 30K membrane (Merck Millipore Ltd Tullagreen, Co. Cork, Ireland) (5,000 *g*, 10 min at 4°C). Endotoxin levels of wtCap and CapVP8* were measured using LAL-based assay Endosafe PTS™ -2005 (Charles River Laboratory, MA, USA). Formulations of CapVP8* in L1-buffer were stored at -80 ºC whereas wtCap and CapVP8* in L2-buffer were used for *in vitro* VLP assembly.

5.2.5. VLP assembly and characterization

In vitro assembly of wtCap and CapVP8* into VLPs was as previously described [35] except dialysis was performed for 24 h. VLP size distribution was analyzed by Asymmetric Flow Field-Flow Fractionation (AF4) coupled with multi-angle light scattering as described previously [46]. Visualization of VLPs with transmission electron microscope (TEM) was also performed as previously reported [46].

5.2.6. VP8* protein preparation

Construct GST-TEVp-VP8* was generated by the Protein Expression Facility (The University of Queensland, Australia). The expression vector was transformed into chemically competent *E. coli* Rosetta™ (DE3) pLysS cells (Novagen, San Diego, CA, USA). Expression of GST-tagged VP8*

was as previously described for expression of GST-tagged VP1 [35] except cultures were induced with 0.1 mM IPTG at 20 ºC for 16 h. Purification of GST-tagged VP8* by affinity chromatography and enzymatic release of the GST tag were performed using published protocols for purification of GST-tagged VP1 [35,39]. After release of GST tag, VP8* protein was recovered from 1.0 mL protein solution with Superdex 75 10/300 GL column (GE Healthcare Biosciences) operated with an AKTAexplorer^{TM} 10 (GE Healthcare Biosciences) liquid chromatography system. The column was pre-equilibrated with PBS at a flow rate of 0.5 mL min^{-1}. Prior to *in vivo* study, endotoxin was removed from protein solution (< 2.5 EU per dose) with anion exchanger as described previously [35].

5.2.7. Dot blotting, immunogold labeling and densitometric analysis

Dot blotting and immunogold labeling were carried out for detection of VP8* antigens from CapVP8* and VLP-VP8* formulations according to the methods reported previously [30] using mouse anti-VP8* monoclonal antibody (RV-5:2) [47]. Proteins were detected and visualized with SDS-PAGE from CapVP8* and VLP- VP8* formulations. Densitometric analysis of the protein bands corresponding to VP1 and modular VP1-VP8* proteins was performed using the BioRad Image lab software version 4.0.1.

5.2.8. Immunization

Four groups of eight female Balb/c mice, aged 6-8 weeks, were obtained from Animal Resources Centre (Murdoch, WA, Australia) and cared for humanely in accordance with the University of Queensland Animal Ethics Committee guidelines. All animal experimental work was reviewed and approved by the University of Queensland Animal Ethics Committee (AIBN/136/15/SMART).

All groups of mice received primary immunizations subcutaneously on Day 0. The first and second booster immunizations were administered subcutaneously 21 and 42 days following primary immunization, respectively. Blood was collected on Days 0, 14, 35 and 56; serum was separated and stored at -20 ºC. Group 1 received 50 µg of unmodified VP1VLP as negative control, Group 2 received 50 µg of CapVP8*/AH adjuvanted with aluminium hydroxide (AH; Alhydrogel®, Brenntag, Germany), Group 3 received 50 µg VLP-VP8*, and Group 4 received 10 µg VP8*/AH as a positive control. The injection volume was 100 µL for all formulations. Adjuvanted formulations were prepared by mixing components together in a 1:1 volume ratio of protein to AH solution and incubation at room temperature for 1 h prior to immunization.

5.2.9. Enzyme-linked immunosorbent assay (ELISA)

Measurement of VP8*- and VP1-specific serum IgG titres by ELISA was as previously described [42] except the 96-well Nunc-Immuno^{TM} MaxiSorp^{TM} plates (NUN442404; Thermo Fisher Scientific, MA, USA) were coated with VP8* or unmodified VP1 VLP antigen at 3.0 µg mL^{-1}, and incubated with CapVP8*/AH and VLP-VP8*, VP8*/AH and unmodified VLP mouse sera initially at 100, 200 and 400-fold dilutions, respectively, followed by four-fold dilutions.

5.2.10. Statistical analysis

Statistical analysis was carried out using GraphPad Prism Version 6.00 (GraphPad Software Inc., CA, USA). Comparison between two groups was performed with unpaired two-tailed t test. Groups were considered significantly different (*) at $p < 0.05$.

5.3. Results and Discussion

5.3.1. Molecular and bioprocess engineering for modular VLPs

Murine polyomavirus VP1 was previously engineered to present small peptide antigenic modules on its surface. A modular capsomere is produced from self-assembly of five modular VP1 monomers *in vivo* in *E. coli*, and a modular VLP is obtained from *in vitro* self-assembly of 72 capsomeres in a controlled cell-free reactor [35,36]. Insertion of a 18 kDa VP8* module derived from human rotavirus outer capsid protein [48] disrupted the structure of capsomeres (unpublished data), suggesting the need to optimize the previously established process and re-design the presentation of large antigenic modules with this murine polyomavirus VLP platform. Insertion of longer linkers and module titration allowed the formation of an insect cell-produced and *in vivo* assembled VLP presenting conformational VP8* module on its surface [30]. A combination of molecular and bioprocess engineering approaches was used here for low-cost production of bacterially-produced and *in vitro* assembled modular VLP-VP8*.

Fig. 5-1 illustrates the molecular engineering of modular capsomere to display a RV 18 kDa VP8* module and its production using low-cost microbial cell factories. A single construct carrying both VP1 and modular VP1-VP8* genes can co-express the proteins in *E. coli*, with the possibility of forming mixtures of non-tagged capsomeres comprising 5VP1, 4VP1:1VP1-VP8*, 3VP1:2VP1-VP8*, 2VP1:3VP1-VP8*, 1VP1:4VP1-VP8* and 5VP1-VP8*. Overexpression of unmodified VP1 decreases the surface density of VP8* module on each pentameric capsomere, thus potentially eliminates steric hindrance to the formation of stable capsomeres. These capsomeres are subsequently extracted and purified to homogeneity for VLP production via *in vitro* assembly.

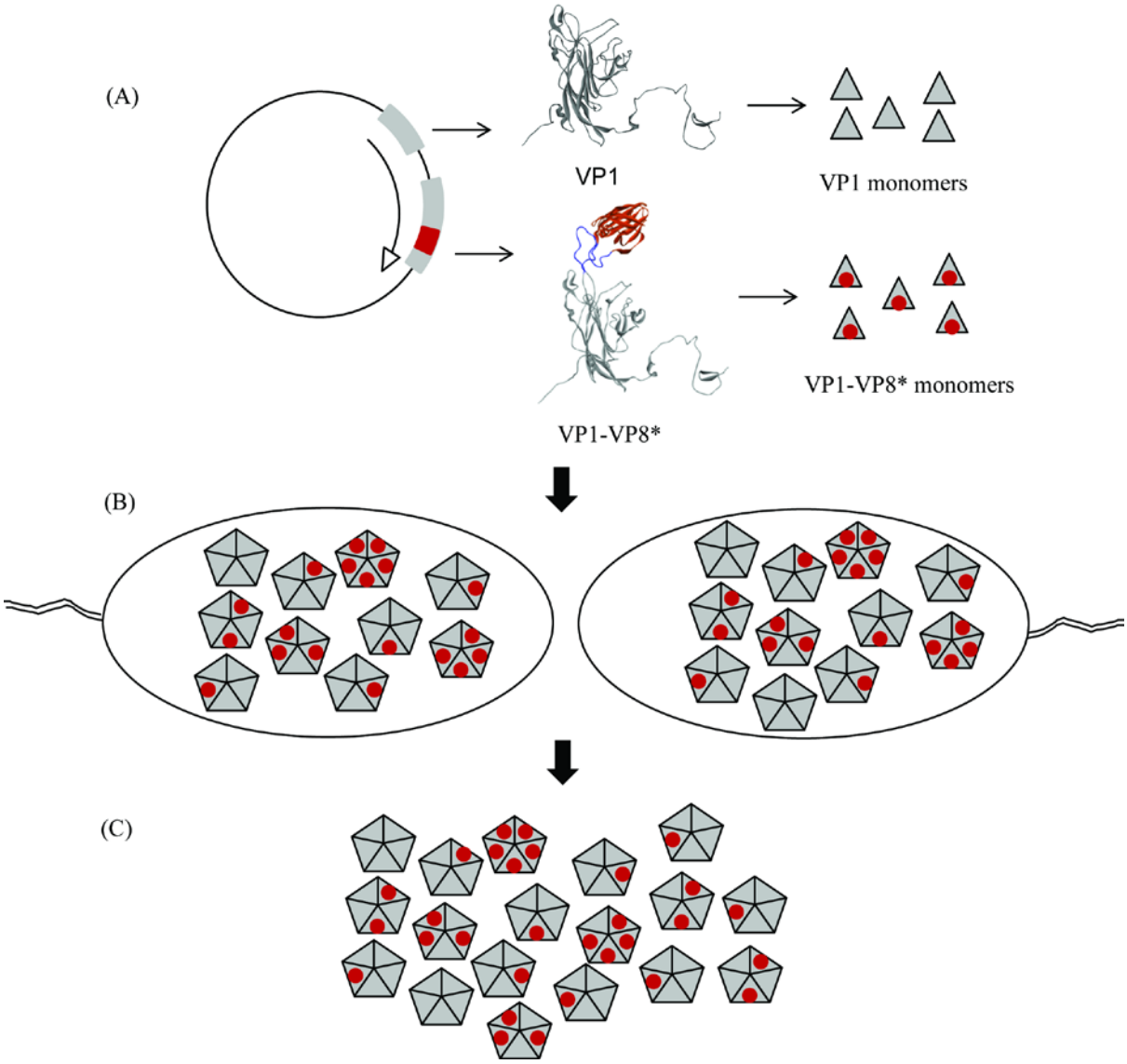

Figure 5-1. Engineering modular capsomere to display a rotavirus 18 kDa VP8 module for Escherichia coli production. (A) A single expression construct for module titration via dual expression of VP1 and modular VP1-VP8* proteins. (B) A mixture of modular capsomeres, made up of VP1 and VP1-VP8* proteins, were obtained via co-expression in Escherichia coli. (C) Recovery of non-tagged modular capsomeres away from Escherichia coli host proteins.*

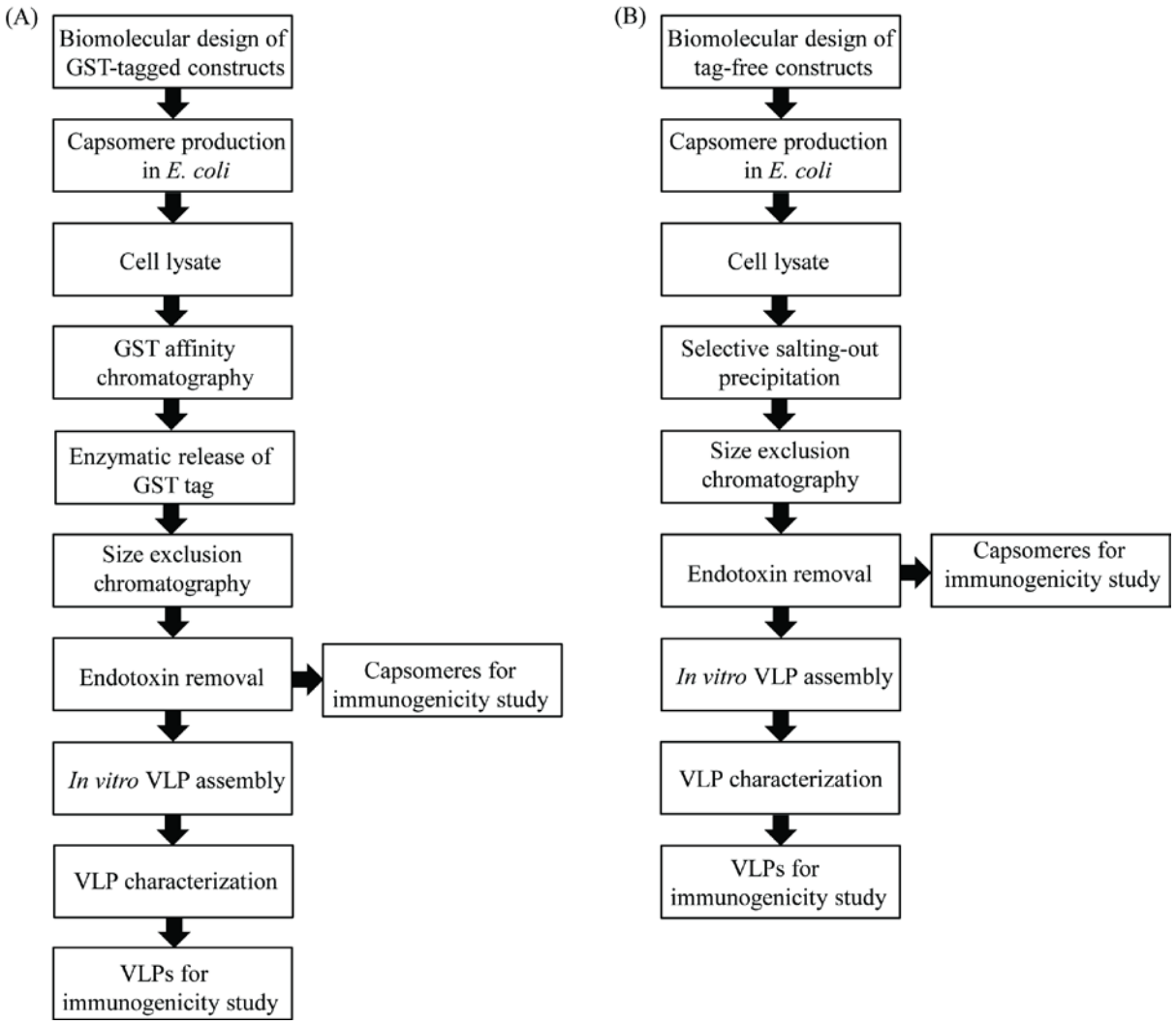

Figure 5-2. Bioprocessing steps for preparation of murine polyomavirus modular virus-like particles (VLPs). (A) Expression and purification of GST-tagged VP1 proteins for production of modular VP1 capsomeres and VLPs using Escherichia coli. (B) A simplified bioprocess for production of non-tagged modular capsomeres and VLPs. Modular VP1 capsomeres were selectively precipitated from Escherichia coli cell lysate for VLP in vitro assembly.

Fig. 5-2 compares the previously established bioprocess with the new simplified bioprocess, for production of murine polyomavirus VP1 VLPs using *E. coli*. To reduce process complexity during modular VP1 protein recovery, the use of GST tag to increase target protein solubility and as purification affinity tag was eliminated. A simple selective salting-out precipitation approach to recover tag-free modular capsomeres from *E. coli* cell lysate was developed in this study (Fig. 5-2B). This approach eliminates affinity chromatography and subsequent enzyme-mediated release of the tag used in the previous process (Fig. 5-2A). Processing of GST-tagged capsomeres adds complexity to the downstream process due to the multimerization effects of GST protein, poor binding affinity and high cost of affinity chromatography media [37]. Release of the GST tag requires treatment with proteases, which adds further cost to the bioprocess [39]. A process simulation study demonstrated that the downstream processing section using chromatography steps

contributed more than two-thirds of total operating cost for capsomere and VLP vaccine production at 10 kL-bioreactor scale [28]. Replacing the chromatography steps with simpler non-chromatographic approaches can reduce process complexity and production cost, and increase the speed and economic benefits of the process [28,42]. Non-chromatographic approaches, such as selective salting-out precipitation, are simple, scalable and amenable to continuous flow operation [42,49]. It can be easily optimized to increase the purity of capsomeres, or coupled with different membrane-based filtration processes [50] to avoid post precipitation polishing chromatographic steps. By employing both molecular and bioprocess engineering, the strategy used in this study has the potential to simplify process complexity and reduce production cost for modular capsomeres and VLPs. This could be particularly attractive to the developing world to get access to safe and effective vaccines at affordable cost, with calculations showing vaccine production at a cost less than 1 cent per dose [28].

5.3.2. Production of stable CapVP8*

Fig. 5-3A shows the co-expression of VP1 and modular VP1-VP8* proteins. The expression of VP1 (42.5 kDa) and VP1-VP8* (64.5 kDa) proteins was detected post-induction. VP1 was overexpressed when co-expressed with VP1-VP8* in *E. coli*. Selective salting-out precipitation of target proteins from *E. coli* cell lysates, with a polishing SEC step (Fig. 5-3B), resulted in the separation of stable modular capsomeres, CapVP8*, from most host cell protein contaminants. As shown by SDS-PAGE analysis (Fig. 5-3C), CapVP8* capsomere peak fraction (Fig. 5-3B, P2) contained mainly the target proteins, VP1 and VP1-VP8*. The aggregate peak fractions (Fig 5-3B, P1) contained mainly the *E. coli* host proteins. Purified CapVP8* was greater than 90% in purity (Fig. 5-3C). Endotoxin removal was conducted on the capsomere samples. Capsomeres with the endotoxin level less than 2.5 EU per dose were ready for injection or for *in vitro* VLP assembly in endotoxin-free environment.

Overexpression of VP1 in comparison to modular VP1-VP8* decreased the number of VP8* modules per capsomere and this may eliminate the steric barrier to the formation of modular VLPs (VLP-VP8*) via *in vivo* assembly [30]. In *E. coli*, higher VP1 expression resulted in the formation of stable CapVP8* with less surface density of VP8* modules. Besides titrating module density on CapVP8*, protein co-expression can enhance solubility of modular VP1-VP8* by enabling proper folding of each protein subunit, thus leading to the formation of soluble and stable complexes, a phenomenon observed for production of protein complexes [51].

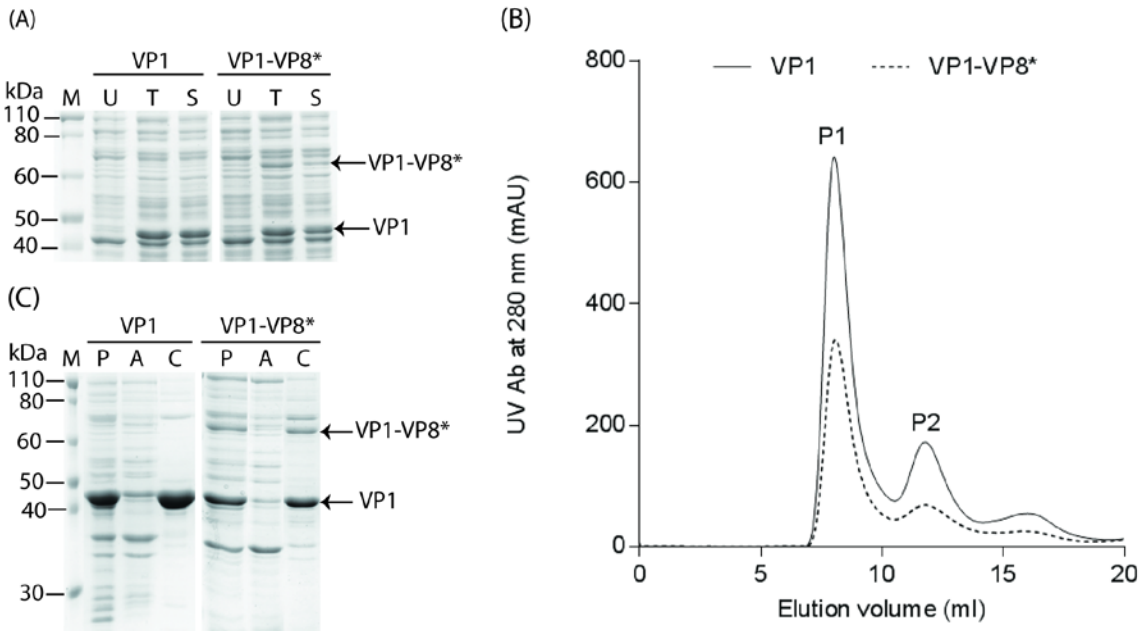

Figure 5-3. Analysis on expression and purification of modular capsomeres. (A) SDS-PAGE analysis on the expression of non-tagged VP1 and modular VP1-VP8 in Escherichia coli. Marker protein (M), un-induced cultures (U), total cell lysate (T) and soluble fraction (S) of induced cultures. (B) Size exclusion chromatograms of capsomeres following selective salting-out precipitation of target proteins. P1 and P2 represent the aggregate and capsomere peaks, respectively. (C) SDS-PAGE analysis on downstream processing of capsomeres. Marker protein (M), purified by selective salting-out precipitation (P), aggregate peak fraction P1 (A) and capsomere peak fraction P2 (C).*

5.3.3. *In vitro* assembly of VLP-VP8* and characterization

Purified modular CapVP8* capsomeres were assembled into VLPs as described in Section 2.5. Fig. 5-4 shows the AF4 fractograms and TEM micrographs of the assembled VLPs, unmodified VP1 VLP (Fig. 5-4A) and VLP-VP8* (Fig. 5-4B). The average root-mean-square (r.m.s) radius for VLP-VP8* was 21.5 nm, almost the same as the radius of unmodified VP1 VLP. The VLPs were stable against aggregation, and they are similar in size distribution and morphology with the corresponding VLPs produced previously [30,35,46].

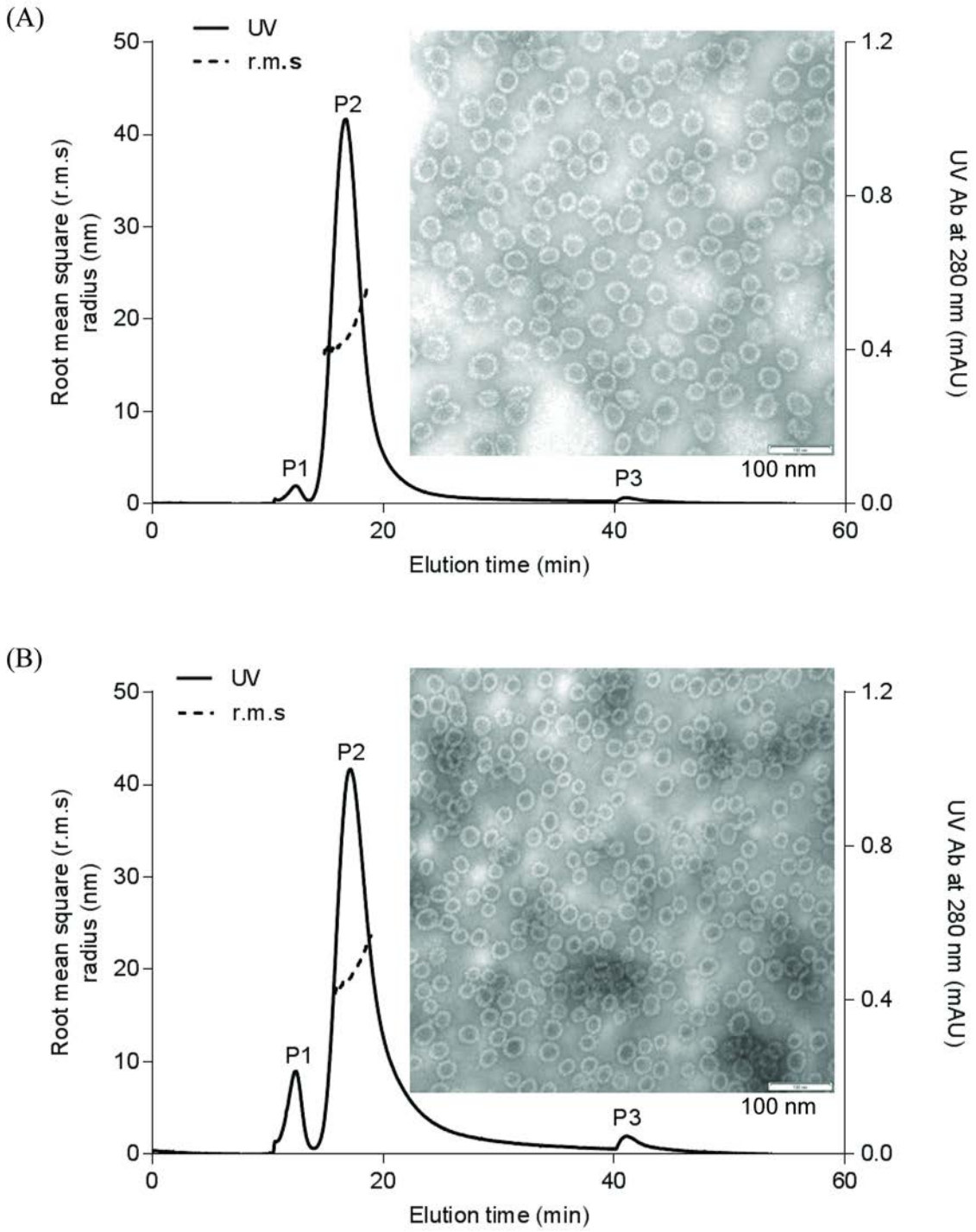

Figure 5-4. Characterization of in vitro assembled VLPs. AF4 fractograms and TEM micrographs of (A) unmodified VLP, and (B) modular VLP-VP8. P1, P2 and P3 represent peaks for unassembled proteins, VLPs and aggregates, respectively.*

Both immunogold labelling of VLP-VP8* (Fig. 5-5B) and dot blot analysis of CapVP8* and VLP-VP8* (Fig. 5-5C) with anti-VP8* monoclonal antibodies confirmed that modularized VP8* is conformational on the surface of purified CapVP8* and *in vitro* assembled VLP-VP8*. These results are in agreement with the results obtained for modular VLP-VP8* produced *in vivo* using a

baculovirus-insect cell co-expression system [30]. Densitometric analysis of the protein bands corresponding to VP1 and VP1-VP8* proteins of CapVP8* and VLP-VP8* samples (Fig. 5-5D) showed that the stoichiometric ratios of VP1 to VP1-VP8* was 4.5:0.5. However, this ratio likely varies between each capsomere and VLP because of the mixed populations of VP1:VP1-VP8* (Fig. 5-1B). While further work is needed to strive for a controlled ratio of VP1 and VP1-VP8* in each capsomere or VLP particle, our results demonstrate the feasibility of module titration using an *E. coli* co-expression strategy for modularization of VLPs with large antigens.

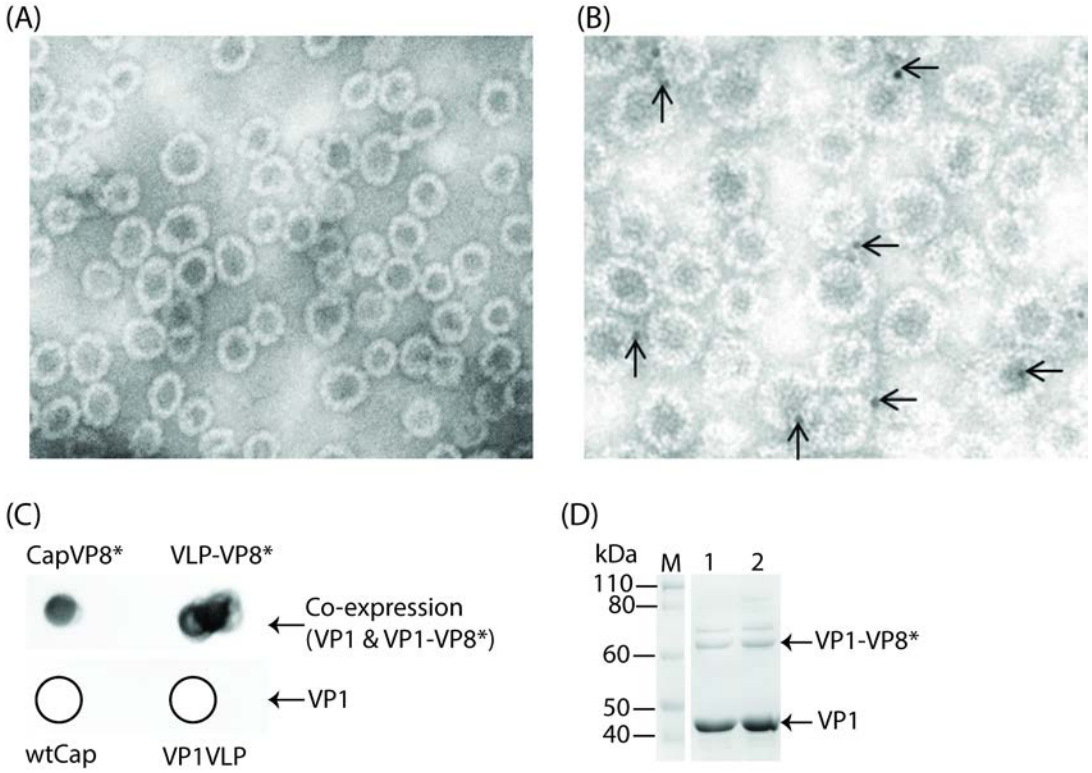

Figure 5-5. Detection of VP8 modules on CapVP8* and VLP-VP8*. (A) TEM micrographs of in vitro assembled VLP-VP8*. (B) Immunogold labelling with anti-VP8* antibodies detected VP8* modules on the surface of in vitro assembled VLP-VP8* (arrows). (C) Dot blot analysis with anti-VP8* antibodies detected VP8* modules in CapVP8* and VLP-VP8* samples. (D) SDS-PAGE analysis of purified CapVP8* and assembled VLP-VP8*, showing the ratio of VP1 and modular VP1-VP8* proteins. Lanes: (M) Marker protein; (1) Purified CapVP8*; (2) In vitro assembled VLP-VP8*.*

5.3.4. Immunogenicity of modular CapVP8* and VLP-VP8*

Modular CapVP8* and VLP-VP8* were evaluated for their immunogenicity in mice. Immunization with 3 doses of CapVP8*/AH and VLP-VP8* each containing approximately 1.5 µg of VP8* antigen per dose elicited high and comparable (P = 0.6730) VP8*-specific IgG titers (~10^5) compared to unmodified VLP (Fig. 5-6A). A strong VP1-specific antibody titre obtained with CapVP8*/AH and VLP-VP8* (Fig. 5-6B) did not interfere with induction of VP8*-specific

antibody. This result is in agreement with the previous study [52,53], indicating that a pre-existing anti-VP1 antibody response does not suppress module-specific immune response. Immunization with 3 doses of VP8*/AH as a positive control produced significantly higher VP8*-specific IgG titre than those elicited by CapVP8*/AH ($P < 0.05$) and VLP-VP8* ($P < 0.01$) (Fig. 5-6A). Production of high levels of VP8*-specific antibody with group immunized with VP8*/AH is likely due to the higher dose of VP8* (10 µg in each dose) in comparison to 1.5 µg of VP8* in each dose of CapVP8*/AH and VLP-VP8*.

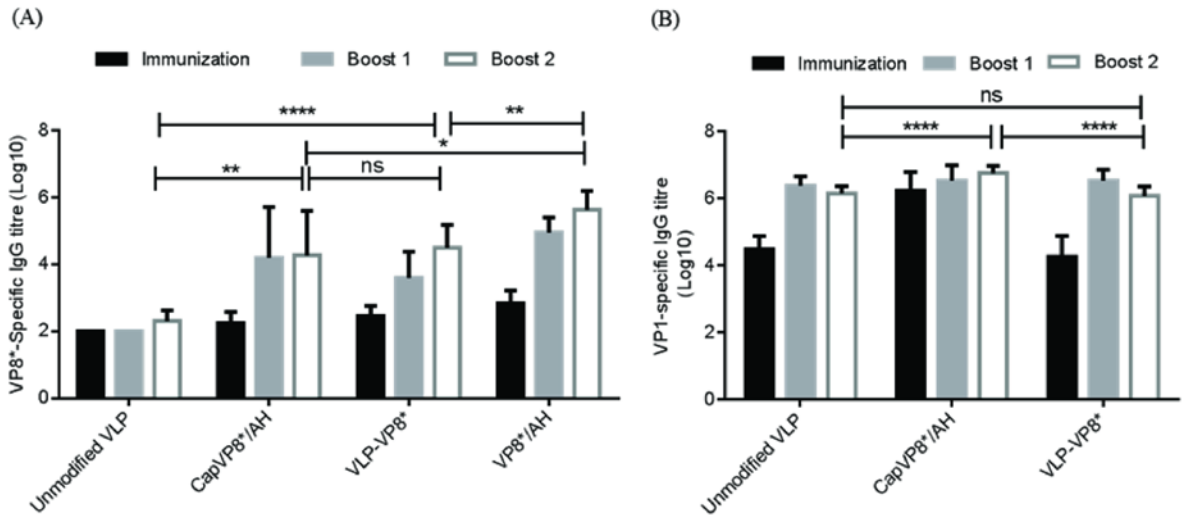

Figure 5-6. VP8- and VP1-specific total IgG titres. (A) VP8*-specific IgG titres induced in mice following three subcutaneous immunizations with CapVP8*/AH, VLP-VP8*, unmodified VLP (as a negative control) and VP8*/AH (as a positive control), separately. (B) VP1-specific IgG titres induced in mice following three subcutaneous immunizations with unmodified VLP, CapVP8*/AH and VLP-VP8*, separately. Statistical analysis of antibody titres after the third immunization (or boost 2) is presented. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ indicate significant difference between the antibody titres of groups, ns indicates no significant differences.*

Production of comparable level of VP8*-specific antibodies against CapVP8* and VLP-VP8* suggested the potential of CapVP8*/AH formulation as alterative cheap vaccine candidate to VLP-VP8*. Previous studies demonstrated that adjuvanted modular murine polyomavirus VP1 capsomeres and human papillomavirus type 16 capsomeres induced almost the same level of immune response as their corresponding VLPs [35,54]. High level of VP8*-specific antibody elicited by VLP-VP8* without adjuvants and previously published results [36] demonstrate the known self-adjuvanting property of VLPs. In addition, the known thermostabilization of VLPs using simple excipients demonstrated for VLPs comprising VP1 [55], also opens the opportunity

for cold-chain elimination and potentially allows low-cost delivery of vaccines for use in the developing world.

5.4. Conclusion

Our aim is to use integrated molecular and bioprocess engineering to simplify production of bacterially-produced modular VLP vaccine candidate against RV, for use in the developing world. Using *E. coli* co-expression strategy of unmodified VP1 and modular VP1, a RV 18 kDa VP8* antigenic module was modularized on murine polyomavirus VP1 capsid protein. Module titration decreased the number of VP8* module on each capsomere, potentially maintaining the stability of modular capsomeres and eliminating steric barrier to VLP assembly. The modular capsomeres, purified from *E. coli* cell lysates by selective salting-out precipitation followed by SEC polishing, formed modular VLPs presenting conformational VP8* modules when assembled *in vitro*. The modular capsomeres (CapVP8*) and VLPs (VLP-VP8*) induced high levels of VP8*-specific antibodies in mice. The results in this study demonstrate that integrating molecular engineering of capsomeres with a simple bioprocessing route allowed the production of highly stable and immunogenic modular capsomeres and *in vitro* assembled modular VLPs using microbial cell factories. This microbial-based VLP vaccine platform can potentially empower the developing world to participate in the development and production of vaccines against RV and other target diseases at affordable cost.

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Conflict of interest statement: The University of Queensland (UQ) filed patents on the use of murine polyomavirus as a vaccine platform. L.H.L.L. and A.P.J.M. contributed to those patents and, through their employment with UQ, hold an indirect interest in this intellectual property. Other authors declare that there are no conflicts of interest.

References

1. Vidya P, Ponnambalam A, Gunasekeran P, Arunagiri K, Sambasivam M, Krishnasami K: **Rota virus infections: prevalence, diagnosis and prevention**. *Journal of Paediatric Sciences* 2015, **7**:e244.
2. Tate JE, Burton AH, Boschi-Pinto C, Steele AD, Duque J, Parashar UD: **2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis**. *Lancet Infectious Diseases* 2012, **12**:136-141.
3. Liu L, Oza S, Hogan D, Perin J, Rudan I, Lawn JE, Cousens S, Mathers C, Black RE: **Global, regional, and national causes of child mortality in 2000–2013, with projections to inform post-2015 priorities: an updated systematic analysis**. *The Lancet* 2015, **385**:430-440.
4. Bagchi P, Nandi S, Chattopadhyay S, Bhowmick R, Halder UC, Nayak MK, Kobayashi N, Chawla-Sarkar M: **Identification of common human host genes involved in pathogenesis of different rotavirus strains: an attempt to recognize probable antiviral targets**. *Virus Research* 2012, **169**:144-153.
5. Glass R, Parashar UD, Bresse J, Turcios R, Fischer TK, Widdowson MA, Jiang B, Gentsch JR: **Rotavirus vaccines: current prospects and future challenges**. *Lancet* 2006, **368**:323-332.
6. Wang CM, Chen SC, Chen KT: **Current status of rotavirus vaccines**. *World Journal of Pediatrics* 2015, **11**:300-308.
7. Angel J, Franco MA, Greenberg HB: **Rotavirus vaccines: recent developments and future considerations**. *Nature Reviews Microbiology* 2007, **5**:529-539.
8. Patel M, Steele D, Gentsch J, Wecker J, Glass R, Parashar U: **Real-world impact of rotavirus vaccination**. *Pediatric Infectious Disease* 2011, **30**:S1-5.
9. Patel M, Shane AL, Parashar UD, Jiang B, Gentsch JR, Glass RI: **Oral rotavirus vaccines: how well will they work where they are needed most?** *Journal of Infectious Diseases* 2009, **200**:S39-S48.
10. Madsen LB, Ustrup M, Fischer TK, Bygbjerg IC, Konradsen F: **Reduced price on rotavirus vaccines: enough to facilitate access where most needed?** *Bulletin of the World Health Organization* 2012, **90**:554-556.
11. Desai R, Curns AT, Patel MM, Parashar UD: **Trends in intussusception-associated deaths among US infants from 1979-2007**. *Journal of Pediatrics* 2012, **160**:456-460.

12. Wen X, Cao D, Jones RW, Li J, Szu S, Hoshino Y: **Construction and characterization of human rotavirus recombinant VP8* subunit parenteral vaccine candidates**. *Vaccine* 2012, **30**:6121-6126.

13. El-Attar L, Oliver SL, Mackie A, Charpilienne A, Poncet D, Cohen J, Bridger JC: **Comparison of the efficacy of rotavirus VLP vaccines to a live homologous rotavirus vaccine in a pig model of rotavirus disease**. *Vaccine* 2009, **27**:3201-3208.

14. Xue M, Yu L, Che Y, Lin H, Zeng Y, Fang M, Li T, Ge S, Xia N: **Characterization and protective efficacy in an animal model of a novel truncated rotavirus VP8 subunit parenteral vaccine candidate**. *Vaccine* 2015, **33**:2606-2613.

15. Settembre EC, Chen JZ, Dormitzer PR, Grigorieff N, Harrison SC: **Atomic model of an infectious rotavirus particle**. *European Molecular Biology Organization Journal* 2011, **30**:408-416.

16. Arias CF, Isa P, Guerrero CA, Méndez E, Zárate S, López T, Espinosa R, Romero P, López S: **Molecular biology of rotavirus cell entry**. *Archives of Medical Research* 2002, **33**:356-361.

17. Favacho ARM, Kurtenbach E, Sardi SI, Gouvea VS: **Cloning, expression, and purification of recombinant bovine rotavirus hemagglutinin, VP8*, in *Escherichia coli***. *Protein Expression and Purification* 2006, **46**:196-203.

18. Lentza EM, Mozgovojb MV, Bellidob D, Dus Santosb MJ, Wigdorovitzb A, Bravo-Almonacida FF: **VP8* antigen produced in tobacco transplastomic plants confers protection against bovine rotavirus infection in a suckling mouse model**. *Journal of Biotechnology* 2011, **156**:100-107.

19. Kovacs-Nolan J, Sasaki E, Yoo DW, Mine Y: **Cloning and expression of human rotavirus spike protein, VP8*, in *Escherichia coli***. *Biochemical and Biophysical Research Communications* 2001, **282**:1183-1188.

20. Andres I, Rodriguez-Diaz J, Buesa J, Zueco J: **Yeast expression of the VP8*fragment of the rotavirus spike protein and its use as immunogen in mice**. *Biotechnology and Bioengineering* 2006, **93**:89-98.

21. Bellido D, Craig PO, Mozgovoj MV, Gonzalez DD, Wigdorovitz A, Goldbaum FA, Santos MJD: **Brucella spp. lumazine synthase as a bovine rotavirus antigen delivery system**. *Vaccine* 2009, **27**:136-145.

22. Wen X, Wen K, Cao D, Li G, Jones RW, Li J, Szu S, Hoshino Y, Yuan L: **Inclusion of a universal tetanus toxoid $CD4^+$ T cell epitope P2 significantly enhanced the immunogenecity of recombinant rotavirus VP8* subunit parenteral vaccines**. *Vaccine* 2014, **32**:4420-4427.

23. Azevedo M, Viasova A, Saif L: **Human rotavirus virus-like particle vaccines evaluated in a neonatal gnotobiotic pig model of human rotavirus disease.** *Expert Review Vaccines* 2013, **12**:169-181.

24. Vieira HLA, Estevao C, Roldao A, Peixoto CC, Sousa MFQ, Cruz PE, Carrondo MJT, Alves PM: **Triple layered rotavirus VLP production: kinetics of vector replication, mRNA stability and recombinant protein production**. *Journal of Biotechnology* 2005, **120**:72-82.

25. Rodriguez-Limas WA, Tyo KEJ, Nielsen J, Ramirez OT, Palomares LA: **Molecular and process design for rotavirus-like particle production in *Saccharomyces cerevisiae***. *Microbial Cell Factories* 2011, **10**:DOI: 10.1186.

26. Palomares LA, Mena JA, Ramirez OT: **Simeltaneous expression of recombinant proteins in the insect cell-baculovirus system: production of virus-like particles**. *Methods* 2012, **56**:389-395.

27. Palomares LA, Ramirez OT: **Challenges for the production of virus-like particles in insect cells: the case of rotavirus-like particles**. *Biochemical Engineering Journal* 2009, **45**:158-167.

28. Chuan YP, Wibowo N, Lua LHL, Middelberg APJ: **The economics of virus-like particles and capsomere vaccines**. *Biochemical Engineering Journal* 2014, **90**:255-263.

29. Zhang L, Lua LHL, Middelberg APJ, Sun Y, Connors NK: **Biomolecular engineering of virus-like particles aided by computational chemistry methods**. *Chemical Society Reviews* 2015, **44**:8608-8618.

30. Lua LHL, Fan Y, Chang C, Connors NK, Middelberg APJ: **Synthetic biology design to display an 18 kDa rotavirus large antigen on a modular virus-like particle** *Vaccine* 2015, **33**:5937-5944.

31. Zhang X, Xin L, Li S, M F, Zhang J, Xia N, Zhao Q: **Lessons learned from successful human vaccines: delineating key epitopes by dissecting the capsid proteins** *Human Vaccines Immunotherapeutics* 2015, **11**:1277-1292.

32. Lua LHL, Connors NK, Sainsbury F, Chuan YP, Wibowo N, Middelberg APJ: **Bioengineering virus like-particles as vaccines**. *Biotechnollogy and Bioengineering* 2014, **111**:425-440.

33. Tan M, Huang P, Xia M, Fang PA, Zhong W, McNeal M, Wei C, Jiang W, Jiang X: **Norovirus P particle, a novel platform for vaccine development and antibody production**. *Journal of Virology* 2011, **85**:753-764.

34. Liew MWO, Rajendran A, Middelberg APJ: **Microbial production of virus-like particle vaccine protein at gram-per-litre levels**. *Journal of Biotechnology* 2010, **150**:224-231.

35. Middelberg APJ, Rivera-Hernandez T, Wibowo N, Lua LHL, Fan Y, Magor G, Chang C, Chuan YP, Good MF, Batzloff MR: **A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines**. *Vaccine* 2011, **29**:7154-7162.

36. Rivera-Hernandez T, Hartas J, Wu Y, Chuan YP, Lua LHL, Good M, Batzloff MR, Middelberg APJ: **Self-adjuvanting modular virus-like particles for mucosal vaccination against group A streptococcus (GAS)**. *Vaccine* 2013, **31**:1950-1955.

37. Lipin DL, Lua LHL, Middelberg APJ: **Quaternary size distribution of soluble aggregates of glutathione-S-transferase purified viral protein as determined by asymmetrical flow field flow fractionation and dynamic light scattering**. *Journal of Chromatography A* 2008, **1190**:204-214.

38. Tekewe A, Connors NK, Sainsbury F, Wibowo N, Lua LHL, Middelberg APJ: **A rapid and simple screening method to identify conditions for enhanced stability of modular vaccine candidates**. *Biochemical Engineering Journal* 2015, **100**:50-58.

39. Connors NK, Wu Y, Lua LHL, Middelberg APJ: **Improved fusion tag cleavage strategies in the downstreamprocessing of self-assembling virus-like particle vaccines**. *Food and Bioproducts Processing* 2014, **92**:143-151.

40. Chuan YP, Lua LHL, Middelberg APJ: **High-level expression of soluble viral structural protein in *Escherichia coli***. *Journal of Biotechnology* 2008, **134**:64-71.

41. Ladd-Effio C, Baumann P, Weigel C, Vormittag P, Middelberg APJ, Hubbuch J: **high-throughput process development of an alternative platform for the production of virus-like particles in Escherichia coli**. *Journal of Biotechnology* 2016, **219**:7-19.

42. Wibowo N, Wu Y, Fan Y, Meers J, Lua LHL, Middelberg APJ: **Non-chromatographic preparation of a bacterially produced single-shot modular virus-like particle capsomere vaccinefor avian influenza**. *Vaccine* 2015, **33**:5960-5965.

43. Laemmli U: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4**. *Nature* 1970, **227**:680-685.

44. Aitken A, Learmonth M: **The protein protocols handbook. In: Walker, J M, editor. Protein determination by UV absorption**. *Springer* 1996:3-6.

45. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A: **The proteomics protocols handbook. In: Walker, J M, editor. Protein identification and analysis tools on the ExPASy server**. *Humana Press* 2005:571-607.

46. Chuan YP, Fan Y, Lua LHL, Middelberg APJ: **Quantitative analysis of virus-like particle size and distribution by field-flow fractionation**. *Biotechnology and Bioengineering* 2008, **99**:1425-1433.

47. Fleming F, Graham K, Taniguchi K, Takada Y, Coulson B: **Rotavirus-neutralizing antibodies inhibit virus binding to integrins alpha 2 beta 1 and alpha 4 beta 1**. *Archives of Virology* 2007, **152**:1087-1101.

48. Monnier N, K H-M, Sun Z, Prasad B, Taniguchi K, Dormitzer P: **High-resolution molecular and antigen structure of the VP8* core of a sialic acid-independent human rotavirus strain**. *Journal of Virology* 2006 **80**:1513-1523.

49. Zhang Z, Chisti Y, Moo-Young M: **Isolation of a recombinant intracellular β-galactosidase by ammonium sulfate fractionation of cell homogenates**. *Bioseparation* 1995, **5**:329-337.

50. Besnard L, Fabre V, Fettig M, Gousseinov E, Kawakami Y, Laroudie N, Scanlan C, Pattnaik P: **Clarification of vaccines: an overview of filter based technology trends and best practices**. *Biotechnology Advances* 2016, **34**:1-13.

51. Kerrigan JJ, Xie Q, Ames RS, Lu Q: **Production of protein complexes via co-expression**. *Protein Expression and Purification* 2011, **75**:1-14.

52. Gedvilaite A, Zvirbliene A, Staniulis J, Sasnauskas K, Krüger D, Ulrich R: **Segments of puumala hantavirus nucleocapsid protein inserted into chimeric polyomavirus-derived virus-like particles induce a strong immune response in mice**. *Viral Immunology* 2004, **17**:51-68.

53. Chuan YP, Rivera-Hernandez T, Wibowo N, Connors NK, Wu Y, Hughes FK, Lua LHL, Middelberg APJ: **Effects of pre-existing anti-carrier immunity and antigenic element multiplicity on efficacy of a modular virus-like particle vaccine**. *Biotechnology and Bioengineering* 2013, **110**:2343-2351.

54. Dell K, Koesters R, Linnebacher M, Klein C, Gissmann L: **Intranasal immunization with human papillomavirus type 16 capsomeres in the presence of non-toxic cholera toxin-based adjuvants elicits increased vaginal immunoglobulin levels**. *Vaccine* 2006, **24**:2238-2247.

55. Mohr J, Chuan YP, Lua LHL, Middelberg APJ: **Virus-like particle formulation optimization by miniaturized high-throughput screening**. *Methods* 2013, **60**:248-256.

Chapter 6

Conclusions and Future Work

6.1. Summary of key research findings

Childhood mortality estimates consistently find diarrhoeal diseases due to RV among the leading causes of deaths [1]. The use of oral live-attenuated RV vaccines (Rotarix™ and RotaTeq®) has reduced childhood mortality in high- and middle-income countries [2-6]. Yet, the burdens of RV and childhood mortality remain unacceptably high, particularly in world´s poorest developing countries because of lower efficacy of the vaccines in the region [7,8]. The use of live-attenuated virus also comes with risk of reversion and safety concerns [9]. In addition, current licensed vaccines suffer from financial and logistic challenges [2,10]. Both Rotarix™ (US$2.5 per dose) and RotaTeq® (US$3.50 per dose) are still too expensive for low-income developing countries even at greatly reduced prices offered by the manufacturing companies [10]. Implementation of RV vaccination in low-income developing countries is also still facing logistic challenges due to the need of the cold chain system and lack of integrated and well-functioning health systems [2,10].

The unresolved challenges with the marketed RV vaccines are the driving factors for development of alternative vaccines for use in the developing world. Low-income developing countries need product development partnerships and technology transfers to stimulate vaccine research, development and manufacturing locally at affordable cost. Developing a simple, efficient, low-cost, rapid and scalable vaccine platform technology can enable developing countries to get RV vaccines at affordable cost from abroad or to manufacture the vaccines locally at home. Therefore, this thesis aims to develop non-replicating RV vaccine candidates using the murine polyomavirus VLP and capsomere platforms for display of RV10 peptide epitope and an 18 kDa large VP8* antigen from a RV spike protein VP8 subunit domain.

The experimental work reported in this thesis was specifically designed to explore three key aspects in the use of the VLP and capsomere platforms for presentation of RV antigenic modules:

(i) Enhance the stability of modular capsomeres. A high-throughput screening (HTS) method was developed to identify buffer additives for enhancing the stability of modular RvVP1 capsomeres presenting three tandem copies of RV10 with polar and charged amino acid residues. The developed screening method allowed the rapid identification of

additives that could enhance the stability of RvVP1 modular capsomeres during downstream processing (Chapter 3).

(ii) Synthetic biology design strategies for RV10 modules. This preliminary study investigated different design options for modules containing a single copy or three tandem copies of RV10, based on synthetic biology, with the aim to address the effect of the hydrophobicity and charge of RV10 containing modules on protein expression and solubility, stability of modular capsomeres, *in vitro* VLP assembly and stability of *in vitro* assembled modular VLPs. Additionally, the effect of reducing the density of RV10 modules on a capsomere surface for *in vitro* assembly of stable modular VLPs was investigated (Chapter 4). The immunogenicity of modular capsomeres and modular VLPs presenting RV10 was also assessed using a mouse model (Appendix B).

(iii) Simplified bioprocess for capsomeres and VLPs presenting a large antigen. An efficient and simple bioprocess was developed through re-engineering of the previously-established bioprocessing steps of the murine polyomavirus VLP platform. The simplified bioprocess allowed production of stable modular CapVP8* and *in vitro* assembled VLP-VP8* presenting a RV 18 kDa VP8* antigenic modules. The preclinical mouse immunogenicity trial was carried out for modular CapVP8* and VLP-VP8* (Chapter 5).

RV10 and VP8* were selected as model RV antigens to develop modular capsomeres and VLPs in this project as they are targets of neutralizing antibodies during RV infection. Previous studies demonstrated that formulations of a modular peptide antigen containing tandem copies of RV10 [11] and VP8*-based subunit vaccines [12] with Freund´s adjuvant elicited a high level of virus neutralizing antibodies.

The following sections describe key findings of this project obtained from experimental work.

6.1.1. Enhance the stability of modular capsomeres

Maintaining the stability of proteins against aggregation remains one of the most challenging tasks in development of safe and effective protein pharmaceuticals and subunit vaccines [13,14]. The poor stability of proteins during downstream processing of RvVP1 modular capsomeres using a stability buffer optimized for unmodified VP1 capsomeres (Chapter 3) was a key challenge to manufacture modular RV VLPs using a murine polyomavirus VLP platform. The specific causes

for poor stability of modular RvVP1 capsomeres are unknown. It was hypothesised that instability of RvVP1 capsomeres may be driven by hydrophobic interactions. Thus, maintaining the stability of RvVP1 capsomeres can necessitate optimization of the buffer conditions during downstream processing.

A HTS method was developed for rapid identification of buffer additives to increase stability of RvVP1. The method, based on DLS analysis to monitor the stability of the capsomeres in the presence of additives, has identified additives that could enhance the stability of RvVP1 capsomeres. Among the various additives tested, non-ionic detergents, TX-100, TW-80 and TW-20, either alone or in combination with L-Arg, were able to enhance the stability of RvVP1. Further analysis with a high-resolution SEC confirmed an improvement in the stability of modular RvVP1 capsomeres in the presence of non-ionic detergents in comparison to those processed using a buffer without any of the three non-ionic detergents. The results in this study highlighted the necessity of tailoring the physicochemical environment specific to each antigenic module for enhanced stability of modular capsomeres. The developed HTS method, based on DLS analysis, is simple, fast and powerful for rapid identification of non-ionic detergents among various other additives able to enhance the stability of modular RvVP1 capsomeres during downstream processing. Non-ionic detergents are very mild chemical compounds that do not denature proteins and they stabilize proteins, suppress aggregation and assist protein refolding [15-20]. They also prevent adsorption-induced protein denaturation and aggregation by competing with proteins for hydrophobic surfaces and interfaces [15,20]. In addition, they may directly interact with hydrophobic regions in protein molecules and reduce their tendency to aggregate [21,22].

Despite their ability in enhancing the stability of modular RvVP1 capsomeres, the use of non-ionic detergents did not increase the purification yield (< 0.5 mg-per-litre of cultures) of RvVP1capsomeres (Chapter 3). RvVP1 capsomeres purified in the presence of TX-100, TW-80 or TW-20 as stabilizing additives did not form modular VLPs when assembled in vitro (Appendix A). The limitation of purification yield after optimization of the downstream processing conditions of RvVP1 capsomeres and requirement for sufficiently stable RvVP1 capsomeres for production of modular VLP via *in vitro* assembly highlight the need for an alternative strategy to improve the stability and purification yield of modular capsomeres. Synthetic biology design strategies of RV10 containing modules may be a suitable approach and thus were pursued in this thesis.

6.1.2. Synthetic biology design strategies for modules

Modularization of the murine polyomavirus VP1 capsomere and VLP platforms may affect expression and solubility of modular proteins, the stability of capsomeres and/or *in vitro* assembly of VLPs depending upon the physicochemical properties and the density of peptide modules on the surface of capsomeres and/or VLPs. As reported in Chapter 3, tailoring the bioprocessing conditions specific to the inserted module was not robust in terms of preparing high yields of RvVP1 capsomeres for further immunogenicity study or for production of VLPs via *in vitro* assembly. Encouraged by the results in Chapter 3, this thesis investigated synthetic biology design strategies for tailoring the physicochemical properties of RV10 modules with the aim of producing high yield of modular capsomeres and VLPs. Using synthetic biology, three modules, $(RV10)_3$, $(RV10)_3$ESE and G4S-Q25-E4-RV10-E4-P6-G4S, were designed and synthesized at the DNA level to examine the effect of the hydrophobicity and charge of modules on protein expression and solubility, stability of modular capsomeres and *in vitro* VLP assembly. Modularization of $(RV10)_3$, $(RV10)_3$ESE and G4S-Q25-E4-RV10-E4-P6-G4S at the DNA level for incorporation into the VLP platform produced VLP-$(RV10)_3$, VLP-$(RV10)_3$ESE and VLP-RV10 modular constructs, respectively. Similarly, modularization of $(RV10)_3$ and $(RV10)_3$ESE into the capsomere platform produced Cap$(RV10)_3$ and Cap$(RV10)_3$ESE constructs, respectively (Chapter 4).

The results in Chapter 4 showed that VLP-$(RV10)_3$ was poorly expressed and no protein was detected for Cap-$(RV10)_3$. The total expression level of proteins from VLP-$(RV10)_3$ESE and Cap-$(RV10)_3$ESE was improved significantly compared to VLP-$(RV10)_3$ and Cap$(RV10)_3$, indicating the contribution of E, ESE and ES residues in enhancing protein expression. A drop in protein solubility was observed for VLP-$(RV10)_3$ESE in comparison to GST-VP1 whereas the solubility was extremely low for proteins from Cap-$(RV10)_3$ESE, showing that protein expression and solubility may also be affected by the insertion site and number of inserted modules. Previous study demonstrated that both the insertion site and the number of inserted modules affected the expression and solubility of GST-tagged capsomeres containing an influenza virus M2e-peptide module [23]. Purification of VLP-$(RV10)_3$ESE modular construct using a stability buffer optimized for purification VP1 capsomeres did not yield stable modular capsomeres. The use of TX-100 enhanced the stability of VLP-$(RV10)_3$ESE modular capsomeres during downstream processing. However, the purification yield was very low and VLP-$(RV10)_3$ESE modular capsomeres did not form modular VLPs via *in vitro* assembly (Chapter 4). The result demonstrated that incorporation of the polar and charged amino acid residues into the module sequence was not sufficient to reduce the hydrophobicity of the module to enhance the stability and purification yield of modular capsomeres.

In addition, the presence of TX-100 might affect inter-capsomere interactions and prevent assembly of capsomeres into VLPs or aggravate aggregation of *in vitro* assembled VLPs.

On the other hand, construct VLP-RV10 expressed good amount of soluble proteins in *E. coli* and resulted in purification of stable modular capsomeres. The expression of soluble protein and the stability of modular capsomeres were improved by reducing copy number of RV10 module and incorporating ionic linkers. The number of RV10 modules was reduced from three tandem copies to one copy with the aim to reduce the stretch of the hydrophobic sequences in $(RV10)_3$ and $(RV10)_3$ESE modules. The ionic linkers, G4S-Q25-E4 and E4-P6-G4S were incorporated in order to reduce the hydrophobicity of the RV10 module by providing additional charges to the hydrophobic RV10 sequence. The longer ionic linkers might also maintain the module structure above the surface of capsomeres and avoid structural perturbations that may compromise the stability of capsomeres and VLPs. Previous study has observed that while modular VP1 inserted with VP8* using a short flexible linker, G4S did not express VLP, incorporation of longer linkers, G4S-Q25 and P6-G4S resulted in formation of stable modular VP8*VLP *in vivo* using a baculovirus-insect cell co-expression system [24]. Structural prediction of modular capsomeres with homology modelling confirmed that the longer linkers were able to maintain the structure of VP8* module above the surface of VLPs [24]. Although high stability of modular capsomeres were observed from construct VLP-RV10 with longer ionic linkers (G4S-Q25-E4 and E4-P6-G4S), the capsomeres did not form modular VLPs via *in vitro* assembly in a cell-free bioreactor (Chapter 4).

In consideration that the presence of two E4 residues in each module might cause strong electrostatic ionic repulsive interactions between capsomeres, preventing assembly of modular capsomeres into VLPs, the density of modules on the surface of capsomeres was titrated down via co-expression of unmodified VP1 and modular VP1-RV10 in *E. coli*. The construct, pET-VP1-RV10 co-expressed soluble unmodified VP1 and modular VP1-RV10 and resulted in high yields of stable modular capsomeres, CapRV10. *In vitro* assembly of CapRV10 resulted in the formation of modular VLPs, RV10VLP with an average root-mean-square (r.m.s) radius of 21.4 nm. RV10VLP is the first bacterially-produced and *in vitro* assembled VLP displaying RV10. The results demonstrate that reducing the hydrophobicity of RV10 modules using synthetic biology design strategies and reducing the density of RV10 modules on the surface of capsomeres via an *E. coli* co-expression strategy facilitated formation of stable RV10VLP via *in vitro* assembly of CapRV10.

Prior to immunogenicity study in mice, bacterial endotoxins were removed from CapRV10 solutions using anion exchange. The negatively-charged bacterial endotoxins were bound to a Q spin column resin whereas CapRV10 solutions were collected in the flow through fraction. The

endotoxin levels in final capsomere solutions were tested using LAL-based assay and were less than 5 EU per dose of injection used in this study. The capsomere solutions were formulated for immunogenicity study and they were also used for production of RV10VLP under endotoxin-free conditions via *in vitro* assembly in a cell-free bioreactor. Endotoxin removal was carried out at the capsomere level to avoid encapsulation of endotoxins inside VLPs during assembly.

Immunizations with 3 doses of 50 µg of CapRV10/AH and 50 µg of RV10VLP elicited low and comparable ($P = 0.2236$) RV10-specific IgG titers (~ $10^{2.5}$ and 10^{3} for CapRV10/AH and RV10VLP, respectively) and the RV10-specific IgG titre elicited by RV10VLP was significantly higher than that elicited by the negative control, unmodified VLP ($P < 0.05$) (Appendix B). Slightly high immunogenicity of RV10VLP in comparison to CapRV10/AH might be associated with higher density of RV10 modules in RV10VLP than CapRV10. Some studies showed that high epitope density effectively enhanced epitope-specific immune response [25] and *in vivo* protective immunity [26]. The titre of RV10-specific antibody induced by RV10VLP however was significantly lower than the level of antibody induced by the recombinant peptide antigen displaying three tandem copies of RV10 [11]. In order to induce potent immune response, formulation of modular capsomeres and VLPs presenting three tandem copies of RV10 might be required. Unfortunately, bioprocessing of modular capsomeres and VLPs from Cap$(RV10)_3$, Cap$(RV10)_3$ESE, VLP-$(RV10)_3$ and VLP-$(RV10)_3$ESE became more challenging because of poor stability of capsomeres, low yield of capsomeres under optimized bioprocessing conditions and failure of these specific modular capsomeres to form VLPs via *in vitro* assembly. Other alternative approaches to improve the immunogenicity of modular capsomeres and VLPs against RV may be based on presentation of large conformational RV antigens on the surface of capsomeres and VLPs. Previous study has reported that a highly stable and immunogenic RV modular VLP vaccine candidate was made inside *E. coli* based on the combination of a RV VP8* antigen and the protrusion (P) domain of the norovirus capsid protein [27]. Lua *et al*. [24] also successfully presented the VP8* antigen on the surface of murine polyomavirus VP1 for production of modular VLP-VP8* using a baculovirus-insect cell co-expression system. However, production of modular VLP-VP8* inside the expression host cells often suffers from the limitations of yield and scalability, complex and expensive bioprocessing steps to commercialize the vaccine candidates for use in the developing world. Therefore, developing an alternative process for an efficient, simple and low-cost manufacturing of modular capsomeres and VLPs presenting VP8* might facilitate further development of the vaccine candidates against RV, particularly for use in the developing world. This idea was covered in Chapter 5.

6.1.3. Molecular and bioprocess engineering for simplified production of modular VLPs presenting a large antigen

Chapter 4 of this thesis showed the possibility of producing stable CapRV10 and RV10VLP by modulating the hydrophobicity of RV10 modules by synthetic biology design strategies and by reducing the density of RV10 modules on the capsomere surface through protein co-expression strategy in *E. coli*. But, CapRV10/AH and RV10VLP containing a single copy of RV10 were weakly immunogenic (Appendix B). Presentation of large conformational antigen, such as a RV 18 kDa VP8* antigen, on the surface of capsomere and VLPs can enhance the immune response directed against the inserted conformational modules. While modularization of small and relatively hydrophilic peptide modules into the murine polyomavirus VLP platform has been well tolerated [28-30], the insertion of large antigen has, to the best of our knowledge, only been recently achieved, via the use of synthetic biology design for incorporation of VP8* antigen with longer linkers [24]. However, the limitations of yield and scalability, process complexity and high cost of manufacturing and processing of VLP-VP8* from a baculovirus-insect cell co-expression system remain major challenges for further development of VLP-VP8* vaccine. This study attempted to use a combination of molecular and bioprocess engineering approaches for simplified production of modular CapVP8* and VLP-VP8* from a low-cost *E. coli* cell factories. CapVP8*/AH and VLP-VP8* were evaluated for their immunogenicity *in vivo* in mice.

The bioprocess used for manufacturing unmodified murine polyomavirus VLP and modular VLPs using the VLP platform previously [28] was re-engineered for production of CapVP8* and VLP-VP8*. The re-engineered bioprocess (Chapter 5), based on non GST-tagged protein co-expression in *E. coli* and purification of capsomeres using a selective salting-out precipitation and SEC, resulted in modular CapVP8* with high purity. This approach is simple and reduces the cost of bioprocessing as it does not require affinity chromatography purification and subsequent enzyme-mediated release of the GST tag, which often add complexity and cost to the platforms used to recover capsomeres [31,32].

After removal of bacterial endotoxins using anion exchange, CapVP8* was formulated as a vaccine candidate. It was also assembled into VLPs *in vitro* using a cell-free bioreactor. Analysis of the assembly products for particle size and particle size distribution using AF4 coupled with a multi-angle light scattering and visualization of particles with TEM revealed the formation of modular VLP-VP8* (r.m.s radius = 21.5 nm). VLP-VP8* was stable against aggregation. Detection of the VP8* antigen with anti-VP8* specific monoclonal antibody using dot blot analysis and immunogold labelling with gold-conjugated secondary antibody demonstrated presentation of

conformational VP8* antigen on the surface of VLP-VP8* (Chapter 5). The result was in agreement with the previous study that revealed presentation of conformational VP8* antigen on the surface of VLP-VP8* assembled *in vivo* using the baculovirus-insect cell co-expression system [24]. In addition, densitometric analysis of the protein bands corresponding to co-expressed unmodified VP1 and modular VP1-VP8* was able to determine the stoichiometric ratio of VP1 and modular VP1-VP8* from which the amount of VP8* was estimated for CapVP8* and VLP-VP8* formulations. The results presented in this study demonstrate that module titration using an *E. coli* co-expression strategy allowed modularization of capsomeres and VLPs with an 18 kDa VP8* module. Moreover, the new simplified bioprocess was simple, efficient and robust for low-cost production of modular CapVP8* and *in vitro* assembled VLP-VP8*.

CapVP8* and VLP-VP8* were evaluated for their *in vivo* immunogenicity in mice compared to unmodified VLP and VP8* antigen as negative and positive controls, respectively. Immunization with 3 doses of 50 µg of CapVP8*/AH and VLP-VP8* elicited comparable ($P = 0.6730$) and high VP8*-specific IgG titres (~10^5). Strong VP1-specific IgG titres (~$10^{6.5}$) did not suppress induction of VP8*-specific immune response against CapVP8* and VLP-VP8* (Chapter 5). These results are consistent with previous study [33,34] indicating the presence of a strong pre-existing anti-VP1 antibody did not interfere with induction of module-specific antibody by modular vaccine candidates. However, the VP8*-specific IgG titres elicited by CapVP8*/AH and VLP-VP8* were significantly lower ($P < 0.05$) than those elicited by VP8*/AH used as positive control. The high immunogenicity of VP8*/AH might be associated with the high amount of VP8* (~ 30 µg per 3 doses) for VP8*/AH formulation that is greater than the amount of VP8* (~ 4.5 µg per 3 doses) for CapVP8*/AH and VLP-VP8* formulations. VLP-VP8* elicited high and comparable levels of VP8*-specific IgG titres like those elicited by CapVP8*/AH, indicating the self-adjuvanting property of VLP-VP8*. This result is in agreement with a previous study that demonstrated self-adjuvanting property of modular VLP presenting J8-peptide modules from group A streptococcus [30]. The *in vivo* immunogenicity result for CapVP8*/AH and VLP-VP8* showed the promising potential of modularization of weakly immunogenic large VP8* antigen into viral capsomeres and VLPs for eliciting high VP8*-specific IgG titre at extremely low dose of VP8*. Interestingly, VLPs with self-adjuvanting property will have huge potential for delivery of adjuvant-free vaccines against RV and other various target diseases.

This study introduces, to the best of our knowledge, the first bacterially-produced *in vitro* assembled and immunogenic VLP-VP8* displaying an 18 kDa large RV VP8* antigen. In addition, the efficiency and simplicity of the new developed bioprocess for production of CapVP8* and VLP-VP8* will facilitate further development of CapVP8* and VLP-VP8* vaccines and other potential

modular vaccine candidates. It will also enable low-income developing countries to participate in vaccine research, development and manufacturing, and to use the final products at affordable cost.

6.2. Overall conclusion and future work

The high global burden of RV [35-37], the unresolved challenge with marketed live-attenuated oral RV vaccines, particularly in the developing regions [7,9,10,38], fluctuations in RV G/P-genotype that also vary by geography and season [39,40] and the emergence of unusual RV strains [39,41,42] have ignited efforts for the development of next-generation RV vaccines for targeting geographically relevant, serotype-specific as well as heterotypic protection. Among the next-generation RV vaccine candidates under development, RLPs have received attention from various academic and public health organizations for their huge potential in inducing strong immune response and good protective efficacy [43-45]. Studies have also demonstrated development of highly stable modular VLPs containing a RV VP8* antigen as vaccine candidates against RV [24,27]. The modular VLPs have also induced strong VP8*-specific antibody response in mice [27]. Production of both RLPs and modular VLPs inside the expression host cell by using recombinant DNA techniques however remains as a major bottleneck for further development of the VLP vaccine candidates. It often suffers from limitations of yield and scalability, and high bioprocessing cost [24,27,46]. Simplifying process scalability and increasing final product yield, while simultaneously reducing manufacturing and processing cost, are very crucial factors for commercialization of VLP-based vaccines against RV, particularly in the developing world. Therefore, this project attempted to develop novel next-generation RV vaccine candidates using simple, low-cost and scalable murine polyomavirus capsomere and VLP platforms.

The murine polyomavirus VP1 capsomere and VLP platforms have demonstrated huge potential for mass-scale production of modular capsomere [23,28] and modular VLP [28,30,33] vaccine candidates. However, depending upon the physicochemical properties, size and the surface density of modules, modularization may affect protein expression and solubility, the stability of capsomeres or *in vitro* VLP assembly, in a way that cannot presently be predicted. Modularization of different designs of hydrophobic RV10 modules into the capsomere and VLP platforms in this thesis affected protein expression and solubility, the stability of capsomeres or *in vitro* VLP assembly. The results in Chapter 3 concluded that tailoring of the solution conditions using non-ionic detergents as additives enhanced the stability of modular RvVP1 capsomeres. But, modular RvVP1 capsomeres did not form VLPs when assembled *in vitro* in the presence of non-ionic detergents as additives (Appendix A). The final purification yield of modular RvVP1capsomeres would also still remain

low to obtain sufficient capsomere materials for *in vivo* immunogenicity study and/or for manufacturing of modular VLPs via *in vitro* assembly.

The use of different synthetic biology design strategies for RV10 modules in Chapter 4 enabled an increase the charge of RV10 module and to insert ionic linkers as flanking elements for stabilization of modular capsomeres and VLPs presenting RV10 modules. In addition, titrating down the density of RV10 modules on the surface of capsomeres via co-expression of unmodified VP1 and modular VP1-RV10 in *E. coli* resulted in highly stable CapRV10 and *in vitro* assembled RV10VLP. Immunization of mice with 3 doses of CapRV10/AH and RV10VLP elicited low and comparable RV10-specific antibody (Appendix B). The immunogenicity of CapRV10/AH and RV10VLP was significantly lower than previously published result for the peptide antigen displaying three tandem copies of RV10 [11]. This result suggested presentation of large conformational RV antigens to enhance the immune response against modular capsomeres and VLPs.

As described in Chapter 5 of this thesis, module titration strategy using an *E. coli* co-expression and re-engineering of the bioprocessing steps of the VLP platform resulted in modular CapVP8* presenting a RV 18 kDa VP8* module with high purity that were able to form modular VLP-VP8* via *in vitro* assembly. The simplified new bioprocess was simple, efficient and cost-effective for production of modular capsomeres and VLPs. Both CapVP8*/AH and VLP-VP8* induced high levels of VP8*-specific antibodies. The results demonstrate the promising potential of CapVP8* and VLP-VP8*, with high stability, purity and immunogenicity, as viable RV vaccine candidate for further development. Interestingly, adjuvant-free VP8*VLP with high VP8*-specific antibody titre presents an ideal promise for further development of a parenterally or sublingually administered prophylactic vaccine against RV infection in the developing world. Before taking CapVP8*/AH and/or VLP-VP8* to clinical trials, further studies are needed to prove the concepts and generate supporting data on some of the following key aspects:

(i) Both CapVP8*/AH and VLP-VP8* induced production of high VP8*-specific antibody levels, suggesting their potential virus neutralization activity and/or protective efficacy. The virus neutralization titres must be determined using an *in vitro* cell-culture-based fluorescent focus reduction neutralization assay. The preclinical protective efficacy of CapVP8*/AH and VLP-VP8* against RV infection must be determined using appropriate animal models. Protection against diarrhoea should also be determined using the neonatal gnotobiotic pig model or the neonatal mice passive protection model.

(ii) An optimum formulation for CapVP8* and VLP-VP8* must be determined for best neutralization activity or protective efficacy and stability. Vaccines as liquid dosage forms are bulk, and need high cost for transportation in distribution, particularly for use in the developing world where there is poor infrastructure. CapVP8* and VLP-VP8* should be formulated as lyophilized dosage forms to eliminate the cold chain system for delivery of the vaccines to the developing regions. Furthermore, different delivery routes must be investigated. There may be potential to develop a sublingual delivery method, enabling simpler vaccine delivery for inducing mucosal immunity and easier administration of the vaccine in comparison to parenteral delivery systems.

(iii) The result in Chapters 4 and 5 of this thesis demonstrated that titrating down the density of RV10 and VP8* modules on the surfaces of capsomeres via *E. coli* co-expression of unmodified VP1 and modular VP1-RV10 or VP1-VP8* enhanced the stability of modular capsomeres and facilitated *in vitro* assembly of modular VLPs. However, formation of capsomeres inside the cell from assembly of co-expressed VP1 and modular VP1 using a single bicistronic vector system is a random, uncontrolled and complex phenomenon. It often results in mixtures of VP1 capsomeres, modular VP1 capsomeres or capsomeres containing both VP1 and modular VP1 monomers at different ratios. Further study on vector engineering is therefore required to reduce the diversity of capsomeres assembled inside the expression host cells during co-expression of unmodified VP1 and modular VP1. Alternatively, the capsomere with the desired stoichiometric ratio of unmodified VP1 and modular VP1 may be purified and separated from mixtures of capsomeres. This also required further study to develop a method for purification and separation of the desired modular capsomeres.

(iv) The bioprocess developed in this thesis (Chapter 5) allowed production of highly immunogenic modular CapVP8* and VLP-VP8* with high purity. However, the use of SEC for separation of capsomeres from misfolded protein aggregates and co-purified *E. coli* proteins still makes the overall process somewhat complex and costly to adopt the bioprocess for manufacturing of vaccines for the developing world. Therefore, further study is required to optimize the selective salting-out precipitation or to develop other non-chromatographic methods, such as filtration, for production of high yield of pure capsomeres and *in vitro* assembled VLPs without using any chromatographic-based purification steps.

In conclusion, the experimental work in this thesis highlights the potential of tailoring of bioprocess conditions, biomolecular design or redesign of modules using synthetic biology, module titration strategy and bioprocess engineering for simple and low-cost production of stable capsomeres and VLPs presenting RV10 or VP8*. The experimental findings in this thesis along with the proof of concepts and data that will be generated from the suggested future works should be extended to manufacturing of modular capsomeres and VLPs presenting a wide range of RV peptide epitopes, RV antigens or chimeric modules containing multiple RV peptide epitopes and/or antigens. The candidates for future studies may include, particularly, presentation of peptide epitopes, antigens, chimeric peptide modules or chimeric antigenic modules derived from the RV VP4, VP7, VP6 and NSP4 proteins on the capsomere and VLP platforms. Such vaccine design and production strategies can allow delivery of safe, effective and low-cost vaccines against geographically and seasonally relevant RV strains at a global scale.

References

1. Liu L, Oza S, Hogan D, Perin J, Rudan I, Lawn JE, Cousens S, Mathers C, Black RE: **Global, regional, and national causes of child mortality in 2000–2013, with projections to inform post-2015 priorities: an updated systematic analysis**. *The Lancet* 2015, **385**:430-440.
2. Angel J, Franco MA, Greenberg HB: **Rotavirus vaccines: recent developments and future considerations**. *Nature Reviews Microbiology* 2007, **5**:529-539.
3. Dennehy PH: **Rotavirus vaccines: an overview**. *Clinical Microbiology Reviews* 2008, **21**:198-208.
4. Glass R, Parashar UD, Bresse J, Turcios R, Fischer TK, Widdowson MA, Jiang B, Gentsch JR: **Rotavirus vaccines: current prospects and future challenges**. *Lancet* 2006, **368**:323-332.
5. Patel M, Steele D, Gentsch J, Wecker J, Glass R, Parashar U: **Real-world impact of rotavirus vaccination**. *Pediatric Infectious Disease* 2011, **30**:S1-5.
6. Vesikari T: **Rotavirus vaccination: a concise review**. *Clinical Microbiology and Infection* 2012, **18**:57-63.
7. Mirzayeva R, Steele AD, Parashar UD, Zaman K, Neuzil KM, Nelsong EAS: **Evaluation of rotavirus vaccines in Asia-are there lessons to be learnt?** *Vaccine* 2009, **27**:F120-F129.
8. Patel M, Shane AL, Parashar UD, Jiang B, Gentsch JR, Glass RI: **Oral rotavirus vaccines: how well will they work where they are needed most?** *Journal of Infectious Diseases* 2009, **200**:S39-S48.
9. Desai R, Curns AT, Patel MM, Parashar UD: **Trends in intussusception-associated deaths among US infants from 1979-2007**. *Journal of Pediatrics* 2012, **160**:456-460.
10. Madsen LB, Ustrup M, Fischer TK, Bygbjerg IC, Konradsen F: **Reduced price on rotavirus vaccines: enough to facilitate access where most needed?** *Bulletin of the World Health Organization* 2012, **90**:554-556.
11. Kovacs-Nolan J, Mine Y: **Tandem copies of a human rotavirus VP8 epitope can induce specific neutralizing antibodies in BALB/c mice**. *Biochimica et Biophysica Acta* 2006, **1760**:1884-1893.
12. Wen X, Cao D, Jones RW, Li J, Szu S, Hoshino Y: **Construction and characterization of human rotavirus recombinant VP8* subunit parenteral vaccine candidates**. *Vaccine* 2012, **30**:6121-6126.
13. Chi EY, Krishnan S, Randolph TW, Carpenter JF: **Physical stability of proteins in aqueous solution: mechanism and driving forces in non-native protein aggregation**. *Pharmaceutical Research* 2004, **20**:1325-1336.

14. Wang W, Nema S, Teagarden D: **Protein aggregation-pathways and influencing factors**. *International Journal of Pharmaceutics* 2010, **390**:89-99.

15. Lee HJ, McAuley A, Schilke KF, McGuire J: **Molecular origins of surfactant-mediated stabilization of protein drugs**. *Advanced Drug Delivery Reviews* 2011, **63**:1160-1171.

16. Bondos SE, Bicknell A: **Detection and prevention of protein aggregation before, during and after purification**. *Analytical Biochemistry* 2003, **316**:223-231.

17. Kragh-Hansen U, Le Maire M, Møller JV: **The mechanism of detergent solubilization of liposomes and protein-containing membranes**. *Biophysical Journal* 1998, **75**:2932-2946.

18. Wang W, Wang YJ, Wang DQ: **Dual effects of tween 80 on protein stability**. *International Journal of Pharmaceutics* 2008, **347**:31-38.

19. Chou D, Krishnamurthy R, Randolph T, Carpenter J, Manning M: **Effects of tween 20 and tween 80 on the stability of Albutropin during agitation**. *Journal of Pharmaceutical Sciences* 2005, **94**:1368-1381.

20. Joshi O, Chu L, McGuire J, Wang DQ: **Adsorption and function of recombinant Factor VIII at the air-water interface in the presence of tween 80**. *Journal of Pharmaceutical Sciences* 2009, **98**:3099-3107.

21. Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB: **Protein-excipient interactions: mechanisms and biophysical characterization applied to protein formulation development**. *Advanced Drug Delivery Reviews* 2011, **63**:1118-1159.

22. Ohtake S, Kita Y, Arakawa T: **Interactions of formulation excipients with proteins in solution and in the dried state**. *Advanced Drug Delivery Reviews* 2011, **63**:1053-1073.

23. Wibowo N, Chuan YP, Lua LHL, Middelberg APJ: **Modular engineering of a microbially-produced viral capsomere vaccine for influenza**. *Chemical Engineering Science* 2012, **103**:12-20.

24. Lua LHL, Fan Y, Chang C, Connors NK, Middelberg APJ: **Synthetic biology design to display an 18 kDa rotavirus large antigen on a modular virus-like particle** *Vaccine* 2015, **33**:5937-5944.

25. Liu W, Chen YH: **High epitope density in a single protein molecule significantly enhances antigenicity as well as immunogenicity: a novel strategy for modern vaccine development and a preliminary investigation about B cell discrimination of monomeric proteins**. *European Journal of Immunology* 2005, **35**:505-514.

26. Liu W, Peng Z, Liu Z, Lu Y, Ding J, Chen YH: **High epitope density in a single recombinant protein molecule of the extracellular domain of influenza A virus M2 protein significantly enhances protective immunity**. *Vaccine* 2004, **23**:366-371.

27. Tan M, Huang P, Xia M, Fang PA, Zhong W, McNeal M, Wei C, Jiang W, Jiang X: **Norovirus P particle, a novel platform for vaccine development and antibody production**. *Journal of Virology* 2011, **85**:753-764.

28. Middelberg APJ, Rivera-Hernandez T, Wibowo N, Lua LHL, Fan Y, Magor G, Chang C, Chuan YP, Good MF, Batzloff MR: **A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines**. *Vaccine* 2011, **29**:7154-7162.

29. Neugebauer M, Walders B, Brinkman M, Ruehland C, Schumacher T, Bertling WM, Geuther E, Reiser COA, Reichel C, Strich S, et al.: **Development of a vaccine marker technology: display of B cell epitopes on the surface of recombinant polyomavirus-like pentamers and capsoids induces pepptide-specific antibodies in piglets after vaccination** *Biotechnology Journal* 2006, **1**:1435-1446.

30. Rivera-Hernandez T, Hartas J, Wu Y, Chuan YP, Lua LHL, Good M, Batzloff MR, Middelberg APJ: **Self-adjuvanting modular virus-like particles for mucosal vaccination against group A streptococcus (GAS)**. *Vaccine* 2013, **31**:1950-1955.

31. Lipin DL, Lua LHL, Middelberg APJ: **Quaternary size distribution of soluble aggregates of glutathione-S-transferase purified viral protein as determined by asymmetrical flow field flow fractionation and dynamic light scattering**. *Journal of Chromatography A* 2008, **1190**:204-214.

32. Connors NK, Wu Y, Lua LHL, Middelberg APJ: **Improved fusion tag cleavage strategies in the downstreamprocessing of self-assembling virus-like particle vaccines**. *Food and Bioproducts Processing* 2014, **92**:143-151.

33. Chuan YP, Rivera-Hernandez T, Wibowo N, Connors NK, Wu Y, Hughes FK, Lua LHL, Middelberg APJ: **Effects of pre-existing anti-carrier immunity and antigenic element multiplicity on efficacy of a modular virus-like particle vaccine**. *Biotechnology and Bioengineering* 2013, **110**:2343-2351.

34. Gedvilaite A, Zvirbliene A, Staniulis J, Sasnauskas K, Krüger D, Ulrich R: **Segments of puumala hantavirus nucleocapsid protein inserted into chimeric polyomavirus-derived virus-like particles induce a strong immune response in mice**. *Viral Immunology* 2004, **17**:51-68.

35. Tate JE, Burton AH, Boschi-Pinto C, Steele AD, Duque J, Parashar UD: **2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis**. *Lancet Infectious Diseases* 2012, **12**:136-141.

36. Liu L, Johansen H, Cousens S, Perin J, Scott S, Lawn J, Rudan I, Campbell H, Cibulskis R, Li M, et al.: **Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000**. *Lancet* 2010, **3**:2151-2161.

37. Lanata C, Fischer-Walker C, Olascoaga A, Torres C, Aryee M, Black R: **Global causes of diarrheal disease mortality in children < 5 years of age: a systematic review**. *PloS One* 2013, **8**:e72788.

38. Gilliland SM, Forrest L, Carre H, Jenkins A, Berry N, Martin J, Minor P, Schepelmann S: **Investigation of porcine circovirus contamination in human vaccines**. *Biologicals* 2012, **40**:270-277.

39. Santos N, Hoshino Y: **Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine**. *Review in Medical Virology* 2005, **15**:29-56.

40. Kim JS, Kim HS, Hyun J, Kim HS, Song W, Lee KM, Shin SH: **Analysis of rotavirus genotypes in Korea during 2013: an increase in the G2P[4] genotype after the introduction of rotavirus vaccines**. *Vaccine* 2014, **32**:6396-6402.

41. Tatte VS, Chitambar SD: **Evidence of discordant genetic linkage in the VP4, VP6, VP7 and NSP4 encoding genes of rotavirus strains from adolescent and adult patients with acute gastroenteritis**. *Infection Genetics and Evolution* 2012, **12**:1630-1634.

42. Matthijnssens J, Bilcke J, Ciarlet M, Martella V, Banyai K, Rahman M, Zeller M, Beutels P, Van Damme P, Van Ranst M: **Rotavirus disease and vaccination: impact on genotype diversity**. *Future Microbiology* 2009, **4**:1303-1316.

43. Azevedo M, Viasova A, Saif L: **Human rotavirus virus-like particle vaccines evaluated in a neonatal gnotobiotic pig model of human rotavirus disease.** *Expert Review Vaccines* 2013, **12**:169-181.

44. El-Attar L, Oliver SL, Mackie A, Charpilienne A, Poncet D, Cohen J, Bridger JC: **Comparison of the efficacy of rotavirus VLP vaccines to a live homologous rotavirus vaccine in a pig model of rotavirus disease**. *Vaccine* 2009, **27**:3201-3208.

45. Jiang B, Estes MK, Barone C, Barniak V, O'Neal CM, Ottaiano A, Madore HP, Conner ME: **Heterotypic protection from rotavirus infection in mice vaccinated with virus-like particles**. *Vaccine* 1999, **17**:1005-1013.

46. Palomares LA, Ramirez OT: **Challenges for the production of virus-like particles in insect cells: the case of rotavirus-like particles**. *Biochemical Engineering Journal* 2009, **45**:158-167.

Appendix A

Characterization of in vitro assembled products of modular RvVP1 capsomeres

Modular RvVP1 capsomeres were purified by SEC in the presence of non-ionic detergents as stabilizing additive in L-buffer described in Section 3.2.7, Chapter 3. After *in vitro* assembly, the capsomeres did not form modular VLPs, as analyzed on TEM (Fig. A2, A3 and A4). VP1 capsomeres were assembled *in vitro* into VLPs as indicated by arrows (Fig. A1). The specific causes preventing modular VLP assembly are unknown. One possibility is the hydrophobic-hydrophobic interactions between surface exposed RV10 elements, driving the formation of aggregates. Thus, surface display of RV10 modules necessitates engineering of the hydrophobicity of the modules to maintain capsomeres stability in compatible VLP assembly buffer. The idea of engineering the hydrophobicity of RV10 modules was covered in Chapter 4 of this thesis.

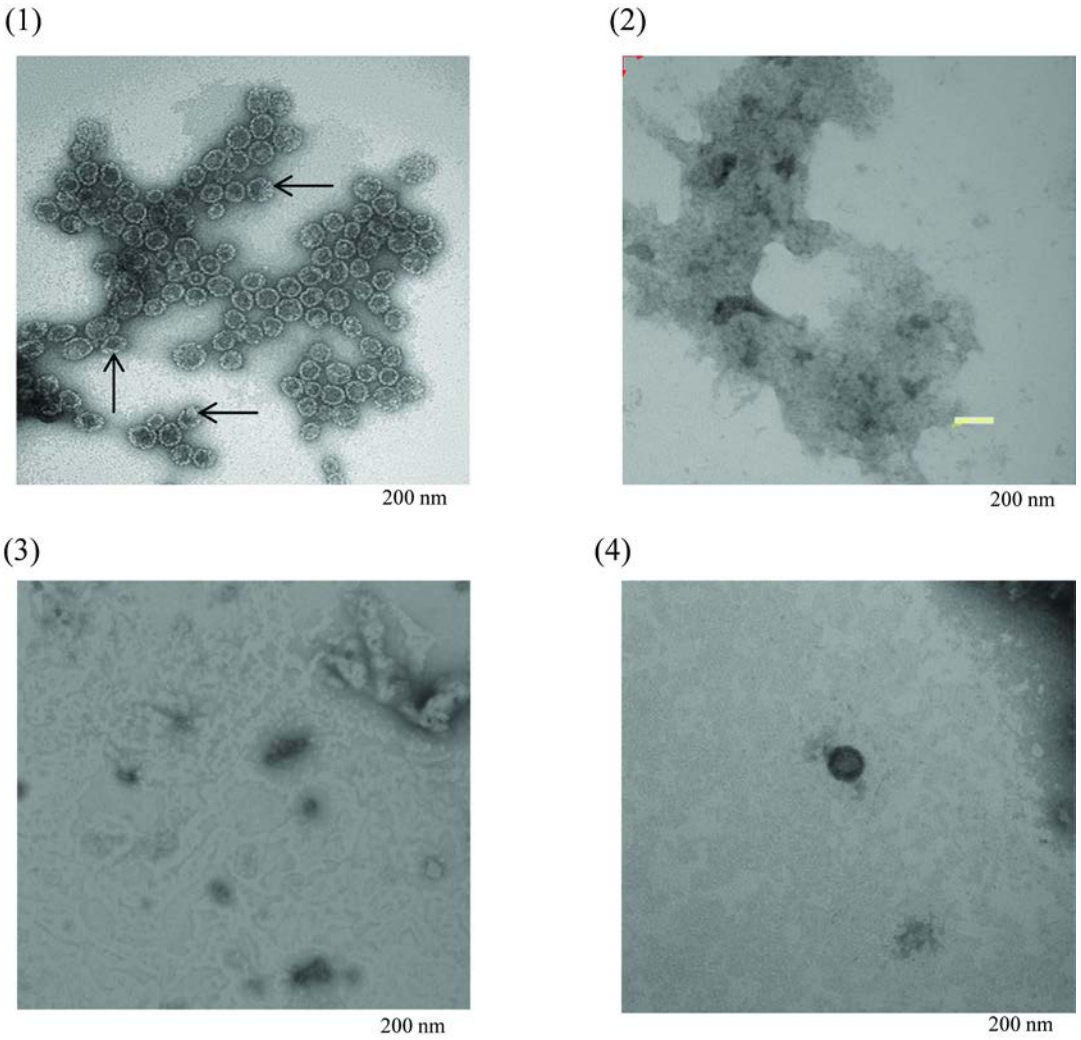

Figure A. Characterization of in vitro assembled products of capsomeres by transmission electron microscope (TEM). TEM micrographs of in vitro assembled products of (1) unmodified VP1 capsomeres purified using TX-100 as buffer additive; (2) RvVP1 capsomeres purified using TX-100 as buffer additive; (3) RvVP1 capsomeres purified using TW-80 as buffer additive; and (4) RvVP1 capsomeres purified using TW-20 as buffer additive.

Appendix B

Immunogenicity of modular CapRV10 and RV10VLP

Modular CapRV10 and RV10VLP produced as reported in Chapter 4 of this thesis were evaluated for their immunogenicity in mice. Balb/c mice immunized with 3 doses of 50 µg of CapRV10/AH and 50 µg of RV10VLP elicited low and comparable (P = 0.2236) RV10-specific IgG titers (~ $10^{2.5}$ and 10^{3} for CapRV10/AH and RV10VLP, respectively). The anti-RV10-specific IgG titer elicited by RV10VLP was significantly higher than that elicited by unmodified VLP used as a negative control (P < 0.05) (Fig. B).

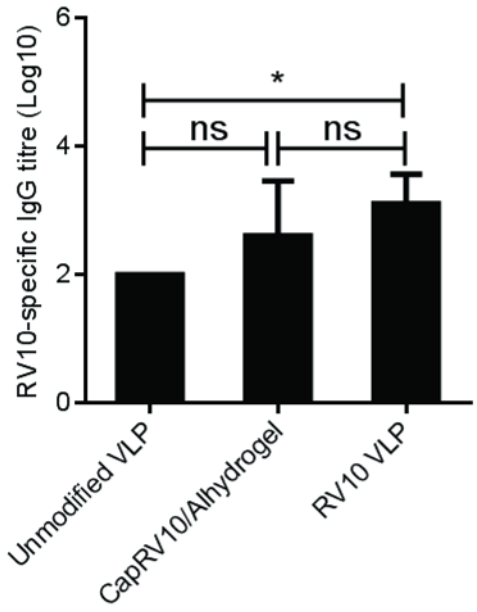

Figure B. RV10-specific total IgG titres. RV10-specific IgG titres induced in mice following three subcutaneous immunizations with CapRV10/AH, RV10VLP, unmodified VLP (as a negative control), separately. Statistical analysis of antibody titres after the third immunization is presented. $^{*}P < 0.05$ *indicates significant difference between the antibody titres of groups, ns indicates no significant differences.*

Previously, Kovacs *et al*. [1] demonstrated that the recombinant peptide displaying a single copy of RV10 induced significantly lower titers of RV10-specific antibody in comparison to a peptide antigen displaying three tandem copies of RV10. These results suggested the need to increase the ratio of antigen with respect to the carrier peptide. Modular capsomere and VLP displaying three tandem copies of RV10 were not included in this study since they were not stable against aggregation as described in Chapter 4. Low RV10-specific immune response against CapRV10 and RV10VLP might be due to low RV10 module to VP1 ratio. RV10 might be also less accessible to

the surface; rather it might be buried into the inner part of the protein to avoid exposure of its hydrophobic amino acids to the solvent and thus, the longer linkers might sterically inhibit binding of B-cell receptor or antibody to RV10. Slightly high immunogenicity of RV10VLP in comparison to CapRV10/AH might be associated with higher density of RV10 modules in RV10VLP than CapRV10. Studies showed that high density M2e-peptide module effectively enhanced M2e-specific immune response [2] and *in vivo* protective immunity [3]. These results suggest the need to tandem display of the RV10 module or other large antigenic modules for effective immune response against RV. Thus the idea of presenting large antigenic modules is covered in Chapter 5.

References

1. Kovacs-Nolan J, Mine Y: **Tandem copies of a human rotavirus VP8 epitope can induce specific neutralizing antibodies in BALB/c mice**. *Biochimica et Biophysica Acta* 2006, **1760**:1884-1893.
2. Liu W, Chen YH: **High epitope density in a single protein molecule significantly enhances antigenicity as well as immunogenicity: a novel strategy for modern vaccine development and a preliminary investigation about B cell discrimination of monomeric proteins**. *European Journal of Immunology* 2005, **35**:505-514.
3. Liu W, Peng Z, Liu Z, Lu Y, Ding J, Chen YH: **High epitope density in a single recombinant protein molecule of the extracellular domain of influenza A virus M2 protein significantly enhances protective immunity**. *Vaccine* 2004, **23**:366-371.